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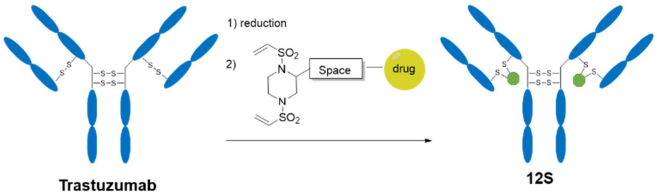


Selective conjugation of the disulfides at Fab regions;
DAR=2;
Highly homogeneous ADCs;
High potency and antigen-selectivity.

Bis(vinylsulfonyl)piperazines as efficient linkers for highly homogeneous antibody-drug conjugates

Rong Huang [a, b, 1], Yao Sheng [a, 1], Ding Wei [a, b], Jianghui Yu [a, b], Hongli Chen [a, *] & Biao Jiang [a, *]

[a] *Shanghai Institute for Advanced Immunochemical Studies, ShanghaiTech University, 393 Middle Huaxia Road, Pudong, Shanghai, 201210, China*

[b] *University of Chinese Academy of Sciences, 19A Yuquan Road, Shijingshan District, Beijing, 100049, China*

** Corresponding author. E-mail addresses: jiangbiao@shanghaitech.edu.cn (B. Jiang), chenhl@shanghaitech.edu.cn (H.L. chen).*

[1] *These authors contributed equally to this work.*

Abstract

Disulfide re-bridging strategy has demonstrated significant advantages in the construction of homogeneous antibody drug conjugates (ADCs). However, a major issue that disulfide scrambling at the hinge region of antibody leads to the formation of "half-antibody" has appeared for many re-bridging linkers. We present bis(vinylsulfonyl)piperazines (BVP) as efficient linkers to selectively re-bridge disulfides at the antigen-binding fragment (Fab) regions and produce highly homogeneous conjugates with a loading of two drugs without disulfide scrambling. We also found that optically active (*S*)-configuration linkers led to more sufficient conjugation compared with (*R*)-configuration. The BVP-linked ADCs demonstrated superior efficacy and antigen-selectivity in vitro cytotoxicity.

Keywords: bis(vinylsulfonyl)piperazines, disulfides re-bridging, antibody drug conjugates, high homogeneity, DAR of two

1 Introduction

Antibody-drug conjugates (ADCs) are targeted therapeutics that combine an antibody with highly potent drugs by chemical linkers. To date, seven ADCs (Adcetris, Kadcyla, Besponsa, Mylotarg, Polivy, Padcev and Enhertu) have been approved for cancer indications and more than 80 ADCs are under clinic trials.[1-4] Pre-clinical and clinical data suggest that ADCs existing as heterogeneous mixtures strongly influence their pharmacokinetics and efficacy. Therefore, efforts have been made for the development of conjugation methods leading to homogeneous ADCs.[5-7] Disulfide

bridging that allows insertion of payloads to inter-chain disulfide bonds of native antibody has emerged as an attractive strategy for the generation of homogeneous ADCs. This approach has shown distinct advantages, including ADCs with high homogeneity, a controlled drug to antibody ratio (DAR), no need of antibody engineering, a general conjugation method for native antibody and retained covalent linkage between light and heavy chains. Bissulfones have been developed as the first class of reagents to cross-link two cysteine residues and construct defined ADCs.[8-10] The resulting ADCs demonstrated high stability and no negative impact on biophysical profile compared to the native antibody. Subsequently, substituted maleimides[11-18] and pyridazinediones[19-25] have been reported as efficient disulfide bridging linkers. Recently, alternatively novel reagents, including 1,3-dichloroacetone,[26] arylenedipropionitrile,[27] thiol-yne coupling reagents,[28] 3,3-bis(bromomethyl) oxetane,[29] hexafluorobenzene,[30] 1,3,5-tris(bromomethyl)benzene[31] and divinylpyrimidine[32] have been developed. These reagents have clearly demonstrated the advantages to the construction of homogeneous ADCs. However, potential disulfide scrambling that disulfide residues cross-link in a non-native fashion has also been observed. Especially for the disulfides at the hinge region, where the two disulfides are close to each other, the released cysteine residues from the disulfides tend to cross-link on the same heavy chain rather than inter-chain fashion, resulting in the production of "half antibody", thus forming a mixture of antibody conjugates (Figure 1a). [15, 27, 32, 33] It is difficult to rule out this incorrect conjugate by optimization of reaction conditions.[32] A "2-in-1" method that incorporated a reduction and a bridging functionalities into a single reagent has been developed to minimize the scrambling product.[21] New reagents are required for the construction of highly homogeneous ADCs without disulfide scrambling products.

Since the incorrect cross-linking conjugate produced from the hinge region of antibody, we hypothesized that selective insertion of drugs from the Fab regions using the strategy of disulfides re-bridging, while the disulfides in the hinge region remained unchanged, highly homogeneous ADCs could be obtained containing two drugs per antibody.

Although an ADC with DAR of four has demonstrated to afford good therapeutic index,[34] a drug loading of two has also presented opportunities for the formation of ADCs with a good efficiency and an improved therapeutic window.[35, 36] To date, this has been addressed with the use of antibody engineering that cysteine residues (THIOMAB)[37] or unnatural amino acids[38] are inserted

on an antibody for site-specific conjugation to afford a conjugate with a loading of two payloads. Engineered approach is complex and costly. Recently, the method that two pairs of the four disulfides on an antibody was "tied-up" has been reported to allow the formation of DAR 2 ADCs without antibody engineering.[25] There is a requirement for more accessible and reliable methods to synthesize ADCs with a loading of two drugs starting from a native antibody.

Our group have been interested in the development of new types of vinyl sulfones for reaction with peptides and proteins.[39-43] Recently, we have reported that divinylsulfonamides are able to achieve site-specific disulfide stapling for peptides.[44] However, application of these reagents to modify complex biomolecules has not been explored. Herein, we describe our development of a novel bis(vinylsulfonyl)piperazine (BVP) linker platform for selective conjugation of disulfide at the Fab regions and the construction of highly homogeneous ADCs with DAR of two (Figure 1b). The resulting ADCs demonstrate superior efficacy and reduced toxicity compared to the FDA approved drug, Trastuzumab Emtasine (T-DM1).

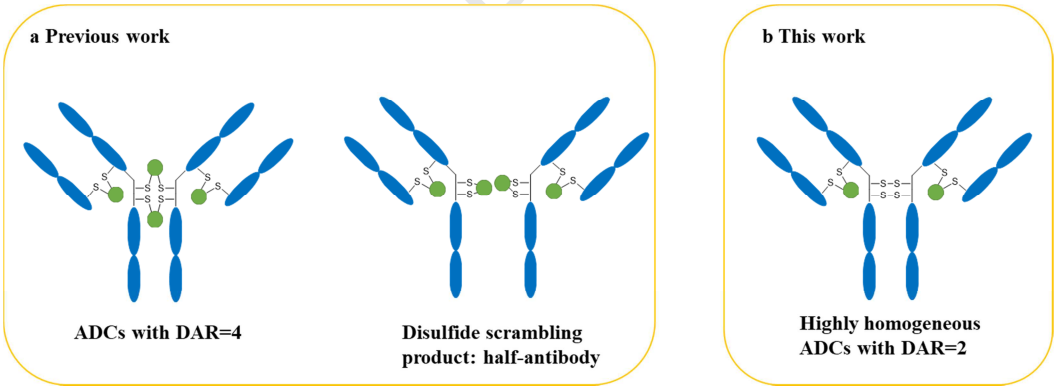
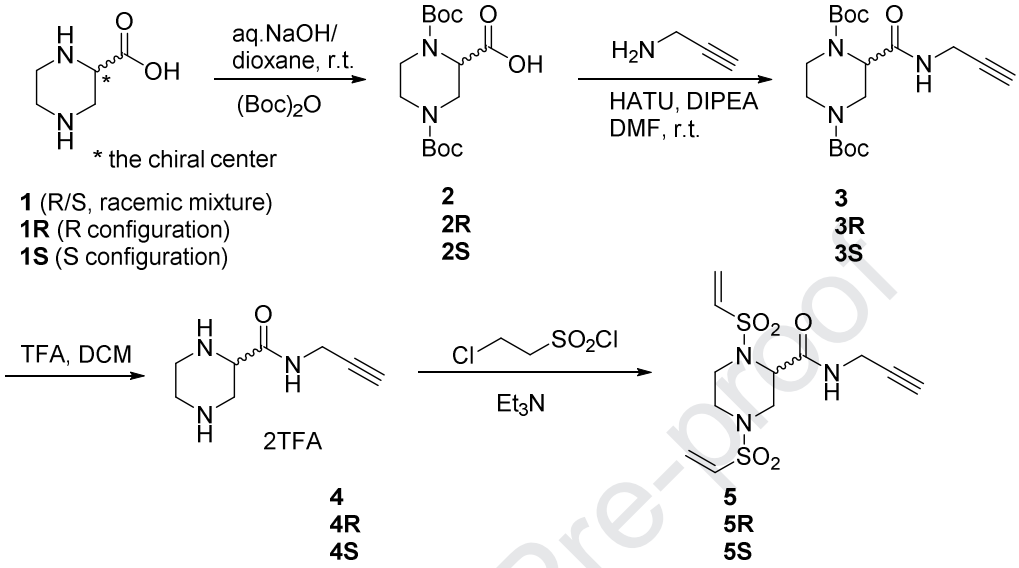


Figure 1 (a) Previous work: the formation of mixture conjugates, (b) This work: highly homogeneous ADCs.

2 Results and discussion

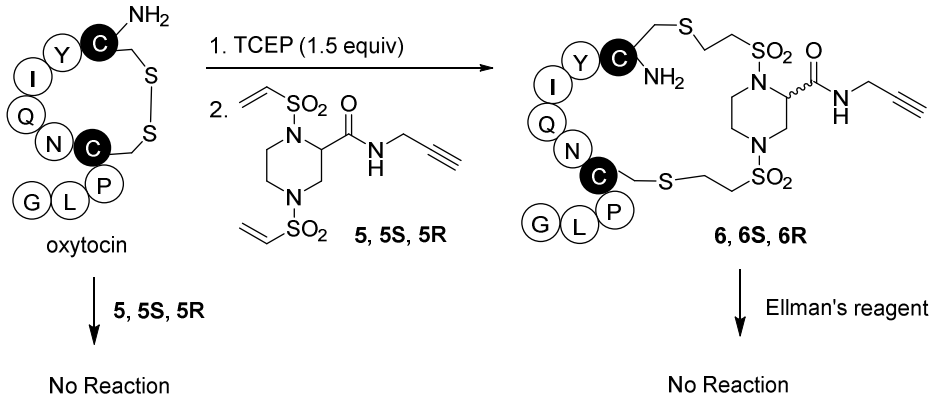
We began our study with the preparation of BVP linkers and identification their potential for cysteine selective re-bridging. BVP linkers, which contain an alkyne functionality for attachment of drugs were synthesized by the route outlined in scheme 1. Initially, racemic piperazine-2-carboxylic acid dihydrochloride (**1**) was protected with di*tert*-butyl dicarbonate to provide the bis-carbamate intermediate **2**, which coupled with propargylamine to afford compound

3. Then this species was deprotected and reacted with 2-chloroethane-1-sulfonyl chloride resulted in the formation of the target linker **5** (Scheme 1). Optically active (*R*)- and (*S*)- linkers (**5R**, **5S**) were also prepared starting from the corresponding (*R*) or (*S*)-piperazine-2-carboxylic acid dihydrochloride (**1S** or **1R**) in the same way.



Scheme 1 Synthesis of bis(vinylsulfonyl)piperazine linkers (BVP) **5**, **5R** and **5S**.

We next investigated the reactivity and selectivity of BVP linkers to react with a peptide, oxytocin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2), which contains a disulfide bond between the Cys^1 and Cys^6. Treatment of oxytocin at pH 7.4 with 1.5 equiv of tris(2-carboxyethyl)phosphine (TCEP) followed by 2.0 equiv of the linker (**5** or **5S** or **5R**) led to conversion to the bridged oxytocin **6** or **6S** or **6R**). Further reaction of these conjugates with Ellman's reagent (5,5′-Dithiobis(2-nitrobenzoic acid) resulted in no reaction, demonstrating that all the cysteine residues were consumed (Scheme 2). No reaction occurred when oxytocin reacted with the linker directly. These results showed that BVP linkers offered the capability to selectively re-bridge disulfide bonds under physiological conditions.



Scheme 2 Reaction of oxytocin with BVP linkers.

Then the BVP linkers **5**, **5R**, **5S** reacted with an azide-functionalized Monomethyl auristatin E (azide-MMAE, **9**), which was produced from the condensation reaction of MMAE (**7**) and an acid **8** *via* click chemistry, respectively to afford BVP-MMAE compounds **10**, **10R** and **10S** (Figure 2a). These compounds were applied to modification of an IgG antibody trastuzumab, which targets HER2 receptor and is an FDA approved drug to treat breast cancer. After the antibody was reduced with 5 equiv of TCEP to release free thiols, **10** or **10R** or **10S** was added to bridge the cysteine residues. To our delight, DAR 2 ADCs, **11** or **11R** or **11S** was produced as the major product, which were confirmed by LC-MS (Figure 2b, Supporting Information Figure S1). What is more, **10S** led to complete conjugation, while a small amount of antibody remains unchanged after conjugation with **10** or **10R** under the same reaction conditions. The conjugates **11**, **11R** and **11S** were further analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The results showed that correctly bridged antibodies were formed and "half antibody" was not found (Figure 2c). The formation of "half antibody" is a recurring issue appeared with many reported re-bridging linkers. Size-exclusion chromatography (SEC) data showed that no obvious aggregation was detected for the conjugates **11**, **11R** and **11S** (Figure 2d).

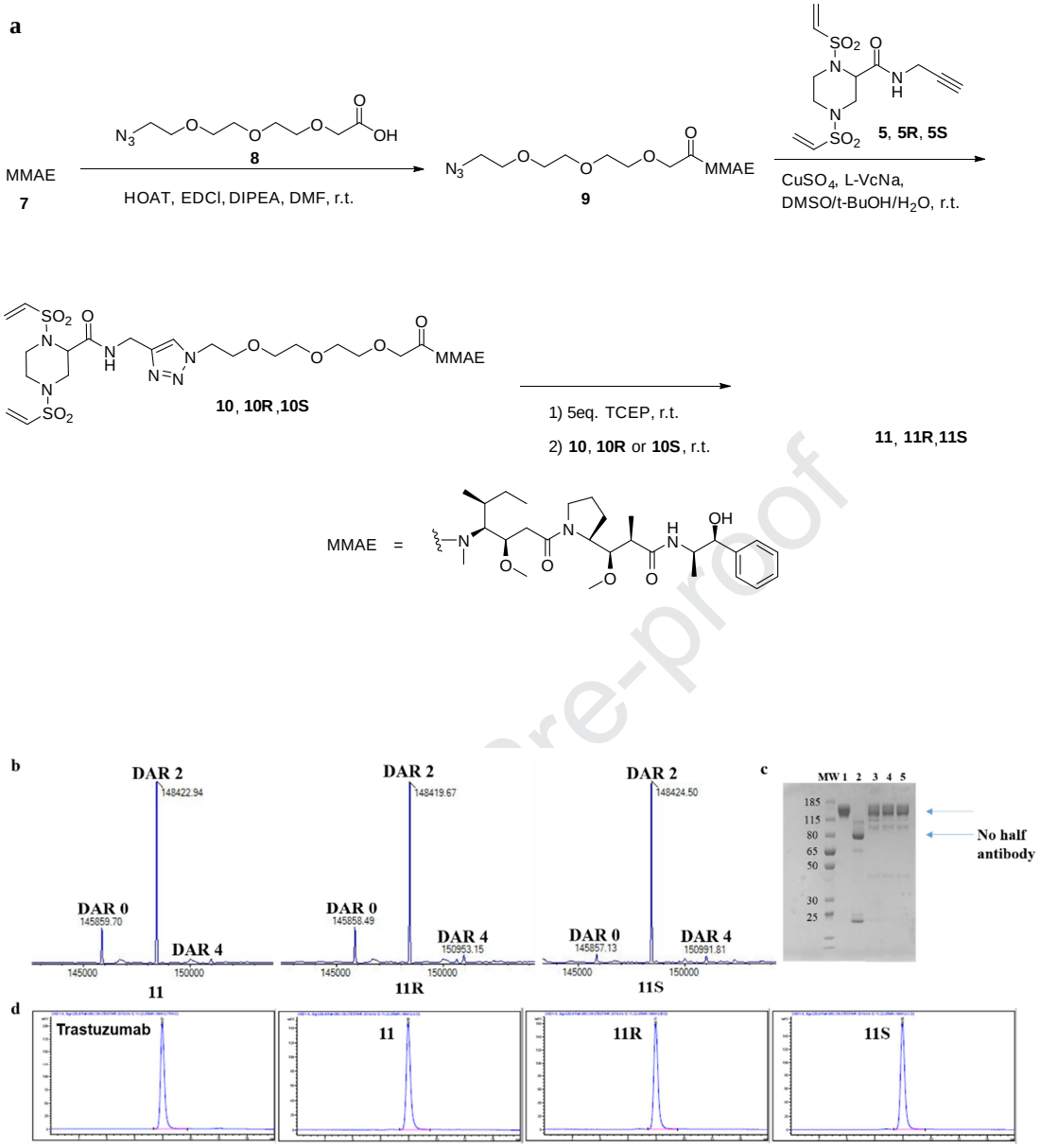


Figure 2 BVP-linked ADCs **11**, **11R** and **11S**. (a) The scheme for the synthesis of **11**, **11R** and **11S**; (b) MS analysis of conjugates **11**, **11R** and **11S**; (c) Analysis of conjugates **11**, **11R** and **11S** by SDS-PAGE; lane 1 for trastuzumab; lane 2 for reduced trastuzumab; lane 3 for **11**, lane 4 for **11R**, lane 5 for **11S** (MW:molecular weight marker); (d) SEC-HPLC analysis of trastuzumab, **11**, **11R** and **11S**.

To further investigate which disulfides were bridged, the conjugates **11**, **11R** and **11S** were digested by papain, which can cleave an antibody into two Fab fragments and one crystalline fragment (Fc). Consistent with the previous report,[17] the papain digestion of trastuzumab cleaved at two sites and generated two types of fragments for Fab. **11**, **11R** and **11S** were also digested under the same condition for trastuzumab. The LC-MS spectra showed that the mass difference

between the Fab ions of conjugates and trastuzumab was equivalent to the mass of one BVP-MMAE compound (**11**, **11R** and **11S**) (Figure 3). Based on the previous results that DAR 2 ADCs were formed, this finding indicates the BVP linkers selectively re-bridged the disulfides at the Fab regions.

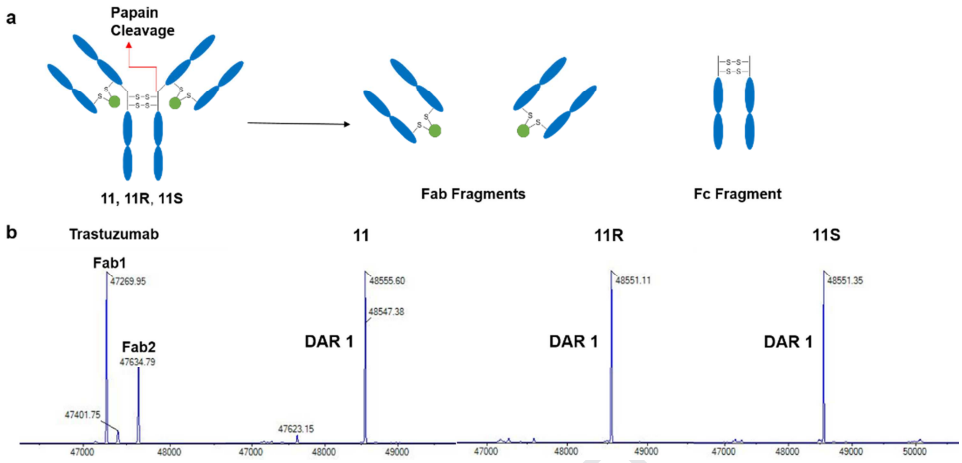


Figure 3 Papain digestion of trastuzumab and the conjugates **11**, **11R** and **11S** . (a) Digestion conditions: papain (250 g/mL of gel), EDTA, Cys*HCl, 20 h, 37 °C, (b) MS spectra for the Fab fragments of **trastuzumab**, **11**, **11R** and **11S** .

To evaluate the stability of BVP conjugates, fluorescent (fluorescein isothiocyanate, FITC) BVP linkers (**12R**, **12S**) and their antibody conjugates **13R** and **13S** were prepared (Supporting Information Scheme S1 and Scheme S2). The in-gel fluorescence and coomassie staining revealed that the conjugates maintained complete resilience to degradation when it was incubated in human plasma at 37 °C for 7 days. When **13R** and **13S** was incubated with 100 equiv of glutathione (GSH), a natural thiol nucleophile at 37 °C, thiol exchange did not occurred because the fluorescence intensity remained without obvious fading (Supporting Information Figure S2 and Figure S3). These results demonstrated that BVP linkers have the capacity to generate highly stable bioconjugates.

We next evaluated the affinity and internalization of the ADCs by ELISA and flow cytometry analysis. The conjugates (**11**, **11R** and **11S**) were found to have retained binding affinity, which was comparable to trastuzumab in both HER2-positive (SK-BR-3) and HER2-negative (MCF7) breast cancer cell lines (Supporting Information Figure S4). Dose-response experiments also showed that BVP linkers had a minimal impact on receptor binding (Figure 4). Flow cytometry was used to monitor internalization of the ADCs in SK-BR-3 cells. The results demonstrated that

all the ADCs had been internalized as efficiently as the native antibody (Figure 5).

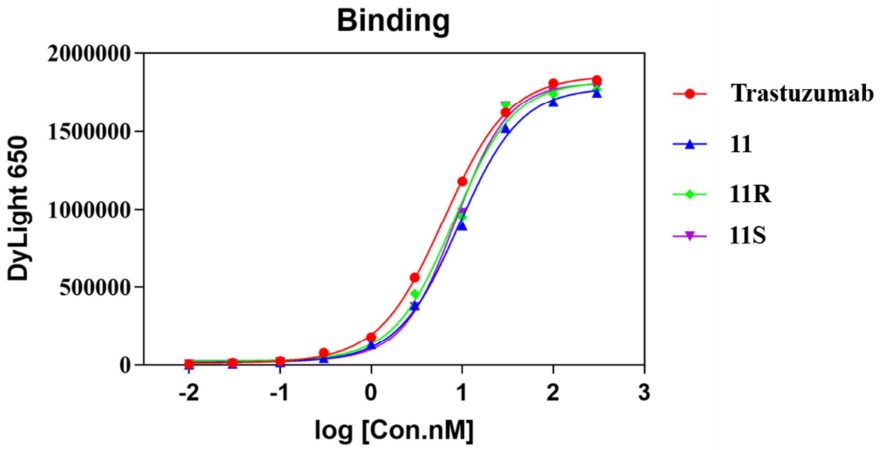


Figure 4 Binding affinity of ADCs and trastuzumab in SK-BR-3 cell via ELISA.

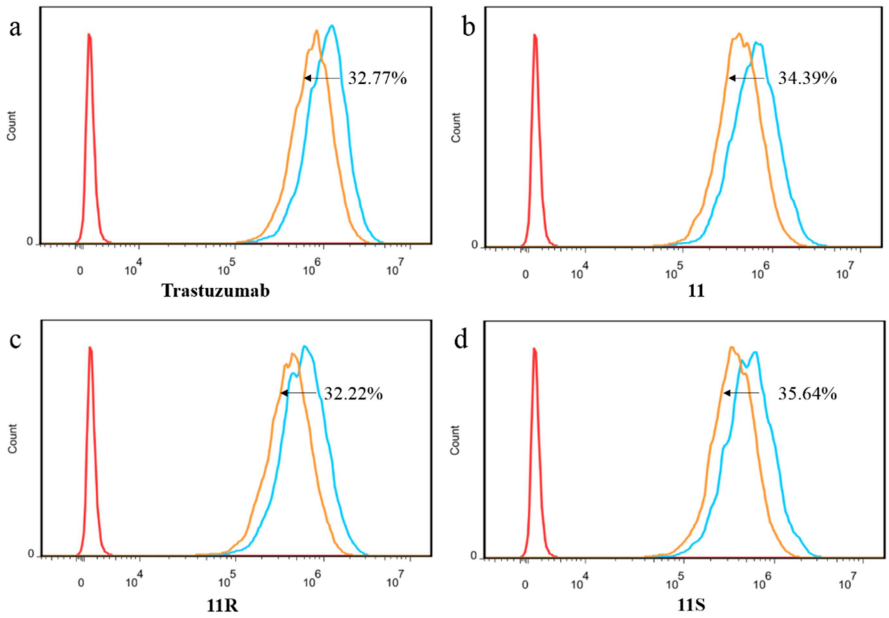


Figure 5 Internalization of ADCs and trastuzumab in SK-BR-3 cell. At the concentration of 100 nmol/L, the percentage of internalization of (a) trastuzumab, (b) **11**, (c) **11R** and (d) **11S** were 32.8%, 34.4%, 32.2% and 35.6% for 3 h, respectively.

The in vitro potency of the conjugates **11**, **11R** and **11S** were examined using the HER2-overexpressing cell lines SK-BR-3 and NCI-N87 as well as the antigen-negative cell lines (MCF7, MDA-MB-231, MDA-MB-468) as controls (Figure 6, Table 1). The ADCs were found to be slightly more potent than T-DM1, which is an approved ADC against the HER2-positive cell lines. Whilst, in the antigen negative cell lines (MDA-MB-231, MDA-MB-468), the conjugates

were less active compared to T-DM1, although all of them demonstrated significantly decreased activities. In contrast to the selective cytotoxicity observed with the conjugates and T-DM1, the free drug MMAE was found to be highly potent and trastuzumab did not cause obvious cytotoxicity in both HER2-positive and HER2-negative cell lines. These results demonstrated that the conjugates were somewhat have a wider therapeutic window than T-DM1.

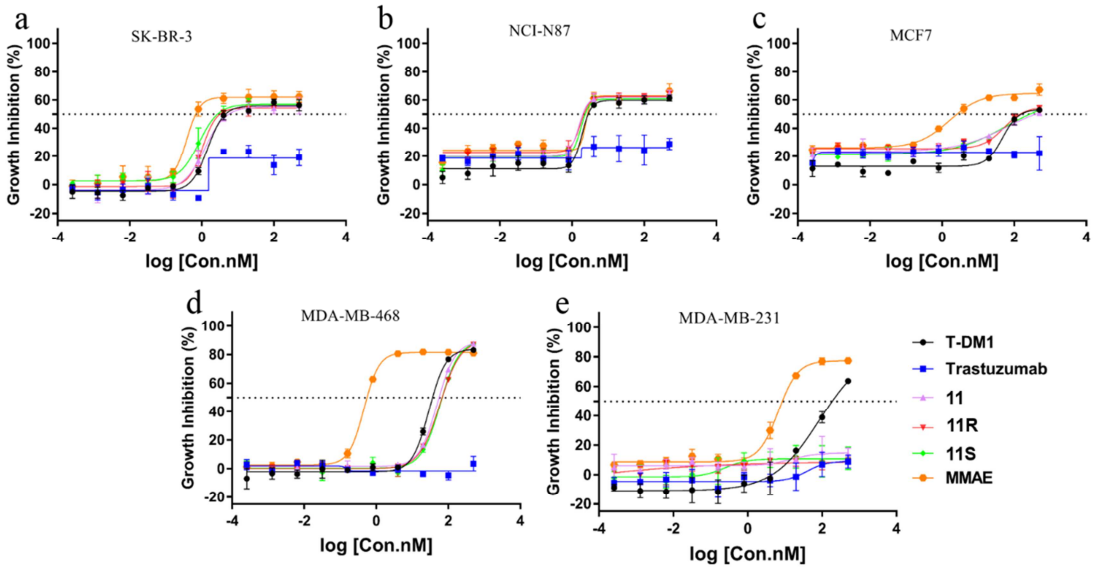


Figure 6 Cytotoxicity of ADCs on different tumor cells including (a) SK-BR-3, (b) NCI-N87, (c) MCF7, (d) MDA-MB-468 and (e) MDA-MB-231.

Table 1 IC_{50} values (nmol/L) of ADCs on different tumor cells (Value = Mean ± SD, n = 3)

Cell lines	**11**	**11R**	**11S**	T-DM1	MMAE	Trastuzumab
SK-BR-3	1.3 ± 0.2	1.2 ± 0.1	0.9 ± 0.4	1.5 ± 0.3	0.4 ± 0.1	> 500
NCI-N87	1.8 ± 0.3	1.9 ± 0.6	1.8 ± 0.5	1.9 ± 0.1	2.0 ± 0.1	> 500
MCF7	34.6 ± 15.2	75.2 ± 20.4	31.9 ± 3.2	59.2 ± 10.2	1.5 ± 0.1	> 500
MDA-MB-231	> 500	> 500	> 500	51.5 ± 15.7	7.7 ± 1.4	> 500

MDA-MB-468	65.7 ± 3.03	77.7 ± 0.2	85.7 ± 6.0	37.7 ± 8.0	0.4 ± 0.01	> 500

3 Conclusions

Disulfide re-bridging strategy has demonstrated significant advantages in the construction of homogeneous ADCs. However, a major issue that disulfide scrambling leads to the formation of "half-antibody" has appeared for many re-bridging linkers. We present novel BVP linkers to selectively re-bridge disulfides at the Fab regions and construct DAR two ADCs without disulfide scrambling. A drug loading of two is preferable for hydrophobic payloads to balance the efficacy and pharmacokinetic profile, such as the PDB-based ADCs.[45, 46] Although genetic engineering of antibodies, such as THIOMABs has worked well to afford ADCs with DAR of two, the strategy with BVP linkers is based on native antibody modification and it is more cost-effective and accessible. Conjugation of MMAE to trastuzumab using the BVP linkers was shown to generate effective ADCs. The conjugates retained the receptor affinity and efficiency of internalization. The ADCs also demonstrated their potency and antigen-selectivity in vitro cytotoxicity and they were found to somewhat have a wider therapeutic window compared with T-DM1. We believe BVP linkers will facilitate the construction of stable and highly homogeneous ADCs with two drug loadings that does not rely on antibody engineering. It is expected that this strategy may find application in other antibody conjugates for therapy and diagnosis.

4 Experimental

4.1 General Procedures

All chemical reagents were of analytical grade, obtained from commercial sources and used as supplied without further purification unless indicated.

Oxytocin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2), which contains a disulfide bond between the Cys^1 and Cys^6 was purchased from Gill Biochemical (Shanghai) Co., Ltd.

Trastuzumab was purchased from Shanghai huanyao biotechnology Co., Ltd. without further purification.

NMR spectra were recorded on a Bruker-500 (500 MHz) instrument. The deuterated solvents employed were purchased from Energy Chemical. Chemical shifts were given in ppm with respect to referenced solvent peaks. Spectra were analyzed with MestReNova.

High-resolution mass spectra (HRMS-ESI) were obtained on an ABsciex 4600 instrument.

HPLC was performed using SHIMADZU LC-30AD. Mobile phases are: 0.5% CF_3COOH on water (solvent A) and acetonitrile (solvent B). *Method:* LC conditions: Agela Technologies C18 column: 2.1X100 mm, 3 μm, column temperature: 30 °C, λ = 254 nm, gradient: 0-10 minutes 10-100% B, 10-12 minutes 100% B, flow rate: 0.4 mL /min.

Optical rotations were measured on an Autopol VI, Serial #91010 Manufactured by Rudolph Research Analytical, Hackettstown, NJ, USA at the indicated temperature with a sodium lamp (D line, 589 nm).

LC-MS was performed using ABsciex 4600. Mobile phases are: 0.1% HCOOH on water (solvent A) and acetonitrile (solvent B). LC conditions: Agela Technologies C18 column: 2.1X100 mm, 3 μm, column temperature: 30 °C, λ = 254 nm, gradient: 0-3 minutes 5-100% B, 3-3.5 minutes 100% B, 3.51-5 minutes 5% B, flow rate: 0.4 mL /min.

Protein LC–MS was performed on ABsciex 4600. Mobile phases are: 0.1% HCOOH on water (solvent A) and acetonitrile (solvent B). LC conditions: GL Sciences C4 column: 2.1X150 mm, 5 μm, column temperature: 70 °C, λ = 254 nm. The gradient was programmed as follows: 0-2.5 minutes 15% B, 2.5-5 minutes 15-95% B, 5-6.5 minutes 95% B, 6.51-8.0 minutes 5% B. The electrospray source was operated with a capillary voltage of 2.0 kV and a cone voltage of 40 V. Nitrogen was used as the desolvation gas at a total flow of 850 L/h. Trastuzumab samples were deglycosylated with Endo S prior to LC-MS analysis.

Size-exclusion chromatography (SEC) was performed using Agilent technologies 1260 Infinity. Mobile phase is Phosphate Buffered Saline (PBS (pH 7.4)). LC conditions: TSKgel G3000SWXL column: 7.8X300 mm, 5 μm, column temperature: 40 °C, λ = 280 nm, gradient: 0-20 minutes 100% PBS (pH 7.4), flow rate: 1 mL /min.

4.2 General procedure of Syntheses

1,4-bis(*tert*-butoxycarbonyl)piperazine-2-carboxylic acid (2). Racemic 2-piperazinecarboxylic acid dihydrochloride (**1**) (0.78 g, 6 mmol) was dissolved in aqueous sodium hydroxide (0.96 g, 40 mmol) solution. Then a solution of di-tert-butyl dicarbonate (3.9 g, 17.9 mmol) in dioxane (25 mL)

was added slowly. The reaction mixture was stirred overnight at room temperature. Subsequently, 1 M hydrochloric acid was added to the reaction system to adjust the pH of the reaction solution to about 4. It was extracted with ethyl acetate (3×20 mL), and the organic phase was combined and dried over anhydrous sodium sulfate. The mixture was concentrated under vacuum to afford 0.88 g (2.67 mmol, 45% yield) **2** as a white solid and used directly in the next reaction. ^{1}H NMR (500 MHz, DMSO-d6) δ 12.73 (s, 1H), 4.43 (m, 1H), 4.32 (m, 1H), 3.83 (m, 1H), 3.66 (m, 1H), 3.05 (m, 2H), 2.78 (m, 1H), 1.38 (s, 18H). ^{13}C NMR (126 MHz, DMSO-d6) δ 171.71, 171.53, 154.91, 154.68, 79.63, 79.45, 79.18, 54.39, 53.07, 27.87. ESI-HRMS Calculated for $C_{15}H_{27}N_2O_6$ $[M+H]^+$:331.1869, Found:331.1872.

di-*tert*-butyl 2-(prop-2-yn-1-ylcarbamoyl)piperazine-1,4-dicarboxylate (3). 1,4-bis(*tert*-butoxycarbonyl)piperazine-2-carboxylic acid (**2**) (0.4 g, 1.21 mmol), 2-propynylamine (0.1 g, 1.8 mmol), [dimethylamino(triazolo[4,5-b]pyridin-3-yloxy)methylidene]-dimethylazanium (0.684 g, 1.8 mmol) and N,N-diisopropylethylamine (0.707 g, 5.4 mmol) were dissolved in N,N-dimethylformamide (5 mL). The reaction mixture was stirred at room temperature overnight followed that water (100 mL) and ethyl acetate (3×50 mL) were added to extract the product. The combined organic extracts were washed sequentially with saturated $NaHCO_3$ (20 mL), NH_4Cl (20 mL) and brine (20 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by column chromatography to afford 0.38 g (1.03 mmol, 85% yield) **3** as a colorless oil. ^{1}H NMR (500 MHz, DMSO-d6) δ 8.44 (s, 1H), 4.25 (m, 2H), 3.73 (m, 4H), 3.32 (m, 1H), 3.28 – 3.04 (m, 2H), 2.83 (m, 1H), 1.36 (s, 18H). ^{13}C NMR (126 MHz, DMSO-d6) δ 169.91, 169.44, 154.70, 153.39, 80.87, 79.33, 78.96, 73.24, 72.90, 54.99, 53.24, 28.05, 27.93. ESI-HRMS Calculated for $C_{18}H_{30}N_3O_5$ $[M+H]^+$:368.2185, Found:368.2172.

***N*-(prop-2-yn-1-yl)piperazine-2-carboxamide (4).** Di-*tert*-butyl 2-(prop-2-yn-1-ylcarbamoyl)piperazine-1,4-dicarboxylate **(3)** (0.38 g, 1.03 mmol) was dissolved in DCM (4 mL). The solution was cooled to 0 ℃ with ice-bath followed by trifluoroacetic acid (1.5 mL) was added, and then the reaction mixture was stirred for 6 hour under ice-bath. The mixture was concentrated under vacuum to afford 0.282 g (1.05 mmol, 98% yield) **4** as a yellow oil and used directly in the next reaction. ESI-HRMS Calculated for $C_8H_{14}N_3O$ $[M+H]^+$:168.1137, Found:168.1135.

***N*-(prop-2-yn-1-yl)-1,4-bis(vinylsulfonyl)piperazine-2-carboxamide (5).** A stirred solution of

N-(prop-2-yn-1-yl)piperazine-2-carboxamide (**4**) (0.282 g, 1.05 mmol) and triethylamine (0.808 g, 8 mmol) in DCM (12 mL) was cooled to 0 °C with ice-bath and 2-chloroethanesµlfonyl chloride (0.652 g , 4.0 mmol) was then injected slowly. The reaction mixture was stirred at 0 °C for 20 minute, and water (30 mL) was added. The product was extracted with DCM (3×20 mL). The combined organic extracts were washed with brine (10 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by column chromatography on a gradient form light petroleum to 50% ethyl acetate in light petroleum to afford 222.34 mg (0.64 mmol, 64% yield) 5 as a slightly yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.54 (t, *J* = 5.5 Hz, 1H), 6.76 (ddd, *J* = 19.0, 16.5, 9.9 Hz, 2H), 6.25 – 6.02 (m, 4H), 4.39 (m, 1H), 4.00 – 3.82 (m, 3H), 3.60 – 3.53 (m, 1H), 3.52 – 3.41 (m, 2H), 3.15 (t, *J* = 2.5 Hz, 1H), 2.92 (dd, *J* = 12.6, 4.3 Hz, 1H), 2.68 (td, *J* = 11.9, 11.4, 3.8 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d6) δ 168.22, 136.09, 133.21, 130.17, 128.10, 81.16, 73.76, 54.79, 47.50, 44.85, 42.26, 28.76. ESI-HRMS Calculated for $C_{12}H_{18}N_3O_5S_2$ [$(M+H)^+$]: 348.0688, found:348.0683.

(*R*)-1,4-bis(*tert*-butoxycarbonyl)piperazine-2-carboxylic acid (2R). (*R*)-2-piperazinecarboxylic acid dihydrochloride (**1R)** (2.0 g, 10 mmol) was dissolved in aqueous sodium hydroxide (1.6 g, 40 mmol) solution. Then a solution of di-*tert*-butyl dicarbonate (4.4 g, 20.2 mmol) in dioxane (25 mL) was added slowly. The reaction mixture was stirred overnight at room temperature. Subsequently, 1 M hydrochloric acid was added to the reaction system to adjust the pH of the reaction solution to about 4. It was extracted with ethyl acetate (3×20 mL), and the organic phase was combined and dried over anhydrous sodium sulfate. The mixture was concentrated under vacuum to afford 2.5 g (7.5 mmol, 77% yield) **2R** as a white solid and used directly in the next reaction. ^{1}H NMR (500 MHz, DMSO-d6) δ 12.73 (s, 1H), 4.43 (m, 1H), 4.32 (m, 1H), 3.83 (m, 1H), 3.66 (m, 1H), 3.05 (m, 2H), 2.78 (m, 1H), 1.38 (s, 18H). ^{13}C NMR (126 MHz, DMSO-d6) δ 171.71, 171.53, 154.93, 154.69, 79.63, 79.46, 79.18, 54.41, 53.09, 27.88. ESI-HRMS Calculated for $C_{15}H_{27}N_2O_6$ $[M+H]^+$:331.1869, Found:331.1872.

(*R*)-di-*tert*-butyl 2-(prop-2-yn-1-ylcarbamoyl)piperazine-1,4-dicarboxylate (3R). (*R*)-1,4-bis(tert-butoxycarbonyl)piperazine-2-carboxylic acid (**2R**) (1.3 g, 3.9 mmol), 2-propynylamine (0.44 g, 7.8 mmol), [dimethylamino(triazolo[4,5-b]pyridin-3-yloxy)methylidene]-dimethylazanium (2.28 g, 6.0 mmol)

and N,N-diisopropylethylamine (2.555 g, 19.5 mmol) were dissolved in N,N-dimethylformamide (10 mL). The reaction mixture was stirred at room temperature overnight followed that water (100 mL) and ethyl acetate (3×20 mL) were added to extract the product. The combined organic extracts were washed sequentially with saturated $NaHCO_3$ (20 mL), NH_4Cl (20 mL) and brine (20 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by column chromatography to afford 1.4 g (3.8 mmol, 95% yield) **3R** as a colorless oil. ^{1}H NMR (500 MHz, DMSO-d6) δ 8.41 (s, 1H), 4.26 (m, 2H), 3.74 (m, 4H), 3.33 (m, 1H), 3.26 – 2.93 (m, 2H), 2.81 (m, 1H), 1.36 (s, 18H). ^{13}C NMR (126 MHz, DMSO-d6) δ 169.89, 169.41, 154.69, 153.39, 80.70, 79.31, 78.91, 73.02, 72.64, 55.00, 53.38, 28.08, 27.90. ESI-HRMS Calculated for $C_{18}H_{30}N_3O_5$ $[M+H]^+$:368.2185, Found:368.2172.

(*R*)-*N*-(prop-2-yn-1-yl)piperazine-2-carboxamide (4R). (*R*)-di-*tert*-butyl-2-(prop-2-yn-1-ylcarbamoyl)piperazine-1,4-dicarboxylate (**3R**) (1.4 g, 3.8 mmol) was dissolved in DCM (5 mL). The solution was cooled to 0°C with ice-bath followed by trifluoroacetic acid (1.5 mL) was added, and then the reaction mixture was stirred for 6 hour under ice-bath. The mixture was concentrated under vacuum to afford 0.6 g (3.57 mmol, 94% yield) **4R** as a yellow oil and used directly in the next reaction. ESI-HRMS Calculated for $C_8H_{14}N_3O$ $[M+H]^+$:168.1137, Found:168.1135.

(*R*)-*N*-(prop-2-yn-1-yl)-1,4-bis(vinylsulfonyl)piperazine-2-carboxamide (5R). A stirred solution of (*R*)-*N*-(prop-2-yn-1-yl)piperazine-2-carboxamide (**4R**) (0.6 g, 3.57 mmol) and triethylamine (3.03 g, 30 mmol) in DCM (12 mL) was cooled to 0 °C with ice-bath and 2-chloroethanesµlfonyl chloride (1.745 g , 10.71 mmol) was then injected slowly. The reaction mixture was stirred at 0 °C for 20 minute, and water (30 mL) was added. The product was extracted with DCM (3×20 mL). The combined organic extracts were washed with brine (10 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by column chromatography on a gradient form light petroleum to 50% ethyl acetate in light petroleum to afford 916 mg (2.64 mmol, 74% yield) **5R** as a slightly yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.54 (t, *J* = 5.5 Hz, 1H), 6.76 (ddd, *J* = 19.1, 16.5, 9.9 Hz, 2H), 6.22 – 6.03 (m, 4H), 4.43 – 4.35 (m, 1H), 3.98 – 3.82 (m, 3H), 3.59 – 3.52 (m, 1H), 3.52 – 3.42 (m, 2H), 3.15 (t, *J* = 2.5 Hz, 1H), 2.92 (dd, *J* = 12.6, 4.3 Hz, 1H), 2.68 (td, *J* = 11.9, 11.4, 3.7 Hz, 1H). ^{13}C

NMR (126 MHz, DMSO-d6) δ 167.74, 135.61, 132.73, 129.69, 127.62, 80.68, 73.28, 54.31, 47.02, 44.37, 41.78, 28.28. ESI-HRMS Calculated for $C_{12}H_{18}N_3O_5S_2$ [$(M+H)^+$]: 348.0688, found:348.0683. $[\alpha]_D^{22.9}$ = - 5.2 (*c* 2.5, CH_3OH)

(*S*)-1,4-bis(*tert*-butoxycarbonyl)piperazine-2-carboxylic acid (2S). (*S*)-2-piperazinecarboxylic acid dihydrochloride (**1S)** (1.0 g, 5 mmol) was dissolved in aqueous sodium hydroxide (0.7 g, 20 mmol) solution. Then a solution of di-*tert*-butyl dicarbonate (2.2 g, 10 mmol) in dioxane (10 mL) was added slowly. The reaction mixture was stirred overnight at room temperature. Subsequently, 1 M hydrochloric acid was added to the reaction system to adjust the pH of the reaction solution to about 4. It was extracted with ethyl acetate (3×20 mL), and the organic phase was combined and dried over anhydrous sodium sulfate. The mixture was concentrated under vacuum to afford 1.15 g (3.5 mmol, 70% yield) **2S** as a white solid and used directly in the next reaction. ^{1}H NMR (500 MHz, DMSO-d6) δ 12.86 (s, 1H), 4.43 (m, 1H), 4.32 (m, 1H), 3.83 (m, 1H), 3.66 (m, 1H), 3.05 (m, 2H), 2.79 (m, 1H), 1.39 (s, 18H). ^{13}C NMR (126 MHz, DMSO-d6) δ 171.71, 171.53, 154.91, 154.67, 79.64, 79.46, 79.18, 54.37, 53.06, 27.86. ESI-HRMS Calculated for $C_{15}H_{27}N_2O_6$ $[M+H]^+$:331.1869, Found:331.1872.

(*S*)-di-*tert*-butyl 2-(prop-2-yn-1-ylcarbamoyl)piperazine-1,4-dicarboxylate (3S). (*S*)-1,4-bis(tert-butoxycarbonyl)piperazine-2-carboxylic acid (**2S**) (1.15 g, 3.5 mmol), 2-propynylamine (0.578 g, 10.5 mmol), [dimethylamino(triazolo[4,5-b]pyridin-3-yloxy)methylidene]-dimethylazanium (1.9 g, 5.0 mmol) and N,N-diisopropylethylamine (2.2 g, 17 mmol) were dissolved in N,N-dimethylformamide (12 mL). The reaction mixture was stirred at room temperature overnight followed that water (100 mL) and ethyl acetate (3×20 mL) were added to extract the product. The combined organic extracts were washed sequentially with saturated $NaHCO_3$ (20 mL), NH_4Cl (20 mL) and brine (20 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by column chromatography to afford 0.83 g (2.26 mmol, 65% yield) **3S** as a colorless oil. ^{1}H NMR (500 MHz, DMSO-d6) δ 8.44 (s, 1H), 4.25 (m, 2H), 3.73 (m, 4H), 3.32 (m, 1H), 3.27 – 3.04 (m, 2H), 2.83 (m, 1H), 1.36 (s, 18H). ^{13}C NMR (126 MHz, DMSO-d6) δ 169.90, 169.44, 154.73, 153.43, 80.85, 79.33, 78.95, 73.27, 72.91, 54.93, 53.26, 28.06, 27.93. ESI-HRMS Calculated for $C_{18}H_{30}N_3O_5$ $[M+H]^+$:368.2185, Found:368.2172.

(*S*)-*N*-(prop-2-yn-1-yl)piperazine-2-carboxamide (4S).

(*S*)-di-*tert*-butyl-2-(prop-2-yn-1-ylcarbamoyl)piperazine-1,4-dicarboxylate (**3S**) (0.83 g, 2.26 mmol) was dissolved in DCM (3 mL). The solution was cooled to 0 °C with ice-bath followed by trifluoroacetic acid (1.5 mL) was added, and then the reaction mixture was stirred for 6 hour under ice-bath. The mixture was concentrated under vacuum to afford 0.378 g (2.26 mmol, 100% yield) **4S** as a yellow oil and used directly in the next reaction. ESI-HRMS Calculated for $C_8H_{14}N_3O$ $[M+H]^+$:168.1137, Found:168.1135.

(*S*)-*N*-(prop-2-yn-1-yl)-1,4-bis(vinylsulfonyl)piperazine-2-carboxamide (5S). A stirred solution of (*S*)-*N*-(prop-2-yn-1-yl)piperazine-2-carboxamide (**4S**) (0.378 g, 2.26 mmol) and triethylamine (2.28 g, 22.6 mmol) in DCM (12 mL) was cooled to 0 °C with ice-bath and 2-chloroethanesµlfonyl chloride (1.474 g , 9.04 mmol) was then injected slowly. The reaction mixture was stirred at 0 °C for 20 minute, and water (30 mL) was added. The product was extracted with DCM (3×20 mL). The combined organic extracts were washed with brine (10 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the residue was purified by column chromatography on a gradient form light petroleum to 50% ethyl acetate in light petroleum to afford 541 mg (1.56 mmol, 69% yield) **5S** as a slightly yellow solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.75 (t, *J* = 5.4 Hz, 1H), 6.64 (dd, *J* = 16.5, 9.8 Hz, 1H), 6.46 (dd, *J* = 16.5, 9.9 Hz, 1H), 6.30 (dd, *J* = 16.5, 14.5 Hz, 2H), 6.07 (dd, *J* = 9.9, 8.1 Hz, 2H), 4.49 (dt, *J* = 3.7, 1.6 Hz, 1H), 4.22 (dt, *J* = 12.8, 1.8 Hz, 1H), 4.17 – 3.93 (m, 2H), 3.67 (m, 2H), 3.28 (ddd, *J* = 13.6, 12.0, 3.5 Hz, 1H), 2.96 (dd, *J* = 12.8, 4.0 Hz, 1H), 2.83 (td, *J* = 12.1, 3.4 Hz, 1H), 2.26 (t, *J* = 2.5 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.22, 135.14, 132.90, 129.73, 128.77, 78.92, 72.17, 55.69, 45.58, 44.24, 42.35, 29.78. ESI-HRMS Calculated for $C_{12}H_{18}N_3O_5S_2$ $[(M+H)^+]$: 348.0688, found:348.0683. $[\alpha]_D^{22.9}$ = + 6.8 (*c* 2.5, CH_3OH)

Compound 9. Monomethyl auristatin E (100.0 mg, 0.14 mmol), 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetic acid (35.6 mg, 0.153 mmol), HOAT (37.9 mg, 0.28 mmol), EDCl (53.5 mg, 0.28 mmol) and diisopropylethylamine (DIEA, 140.6 mg, 1.39 mmol) were dissolved in DMF (5 mL). The reaction mixture was stirred at room temperature overnight. The resulting crude mixture was purified by HPLC to afford 111 mg (0.1189 mmol, 85.6%) yield of 9 as a white solid. ESI-HRMS Calculated for $C_{45}H_{77}N_8O_{10}$ $[M+H]^+$:933.6025, Found:933.5729.

Compound 10. MMAE-3-PEG-N_3 (**9**) (30.0 mg, 0.032 mmol), **5** (12 mg, 0.035 mmol),

Na-ascorbate (12.6 mg, 0.064 mmol) and $CuSO_4$ (10 mg, 0.064 mmol) were dissolved in tBuOH/H_2O/DMF (1/1/1, 3 mL). The reaction mixture was stirred at room temperature for 3 h. The resulting crude mixture was purified by HPLC to afford 24.0 mg (0.01874 mmol, 53%) yield of **10** as a white solid. ^{1}H NMR (500 MHz, Methanol-d4) δ 8.27 – 8.09 (m, 1H), 7.43 – 7.17 (m, 5H), 6.78 – 6.67 (m, 1H), 6.61 (m, 1H), 6.28 – 6.02 (m, 4H), 4.79 – 4.41 (m, 9H), 4.41 – 4.03 (m, 5H), 4.00 – 3.82 (m, 3H), 3.82 – 3.49 (m, 13H), 3.51 – 3.23 (m, 13H), 3.23 – 3.09 (m, 2H), 3.09 – 2.92 (m, 4H), 2.91 – 2.66 (m, 1H), 2.58 – 2.36 (m, 1H), 2.36 – 2.18 (m, 2H), 2.17 – 1.67 (m, 5H), 1.66 – 1.50 (m, 1H), 1.41 (d, *J* = 3.7 Hz, 2H), 1.32 – 0.70 (m, 24H) ^{13}C NMR (126 MHz, Methanol-d4) δ 174.03, 170.08, 169.30, 142.71, 135.51, 132.38, 128.99, 128.13, 127.86, 127.23, 127.10, 126.90, 126.72, 126.58, 82.05, 75.91, 70.42, 70.05, 69.95, 69.15, 68.33, 60.61, 60.23, 59.54, 59.20, 56.94, 55.28, 54.71, 51.36, 49.93, 48.13, 47.96, 47.90, 47.79, 47.73, 47.62, 47.53, 47.45, 47.36, 47.28, 47.11, 46.68, 44.74, 44.43, 44.13, 42.10, 33.77, 31.70, 30.50, 29.02, 26.27, 25.62, 25.20, 24.48, 24.21, 23.07, 18.37, 17.79, 17.36, 14.69, 14.44, 13.79, 9.53. ESI-HRMS Calculated for $C_{59}H_{98}N_{11}O_{16}S_2$ $[M+H]^+$:1280.6634, Found:1280.6644. HPLC analysis: retention time = 4.947 min; peak area, 99%

Compound 10S. MMAE-3-PEG-N_3 (**9**) (48 mg, 0.052 mmol), **5S** (18 mg, 0.052 mmol), Na-ascorbate (20 mg, 0.105 mmol) and $CuSO_4$ (18 mg, 0.105 mmol) were dissolved in tBuOH/H_2O/DMF (1/1/1, 3 mL). The reaction mixture was stirred at room temperature for 3 h. The resulting crude mixture was purified by HPLC to afford 18 mg (0.014 mmol, 27%) yield of **10S** as a white solid. ^{1}H NMR (500 MHz, Methanol-d4) δ 7.96 (s, 1H), 7.43 – 7.17 (m, 5H), 6.80 – 6.51 (m, 2H), 6.27 – 5.98 (m, 4H), 4.72 – 4.50 (m, 8H), 4.50 – 4.03 (m, 8H), 4.03 – 3.80 (m, 3H), 3.80 – 3.49 (m, 13H), 3.49 – 3.24 (m, 13H), 3.24 – 3.08 (m, 2H), 3.06 – 2.71 (m, 6H), 2.58 – 2.41 (m, 2H), 2.39 – 1.51 (m, 6H), 1.31 – 1.08 (m, 3H), 1.08 – 0.69 (m, 21H). ^{13}C NMR (126 MHz, Methanol-d4) δ 173.17, 170.45, 144.01, 143.74, 136.73, 133.73, 130.43, 129.52, 129.25, 128.62, 128.40, 128.06, 127.92, 83.47, 77.33, 71.72, 71.43, 71.41, 70.54, 70.17, 63.69, 62.01, 61.60, 61.54, 60.75, 60.57, 58.64, 58.37, 56.62, 51.51, 51.32, 49.51, 49.34, 49.17, 49.00, 48.83, 48.66, 48.49, 48.08, 46.10, 45.84, 45.49, 43.45, 35.94, 31.82, 30.38, 27.66, 27.59, 26.96, 26.57, 25.80, 25.60, 24.43, 20.89, 19.73, 19.12, 18.97, 18.72, 16.32, 16.09, 15.80, 15.05, 14.45, 10.90. ESI-HRMS Calculated for $C_{59}H_{98}N_{11}O_{16}S_2$ $[M+H]^+$:1280.6634, Found:1280.6684. HPLC analysis: retention time = 5.075 min; peak area, 99%.

Compound 10R. MMAE-3-PEG-N_3 (**9**) (46.0 mg, 0.05 mmol), **5R** (17 mg, 0.05 mmol), Na-ascorbate (19 mg, 0.1 mmol) and $CuSO_4$ (16 mg, 0.1 mmol) were dissolved in tBuOH/H_2O/DMF (1/1/1, 3 mL). The reaction mixture was stirred at room temperature for 3 h. The resulting crude mixture was purified by HPLC to afford 20.0 mg (0.016 mmol, 32%) yield of **10R** as a white solid. ^{1}H NMR (500 MHz, Methanol-d4) δ 8.00 (d, J = 11.8 Hz, 1H), 7.45 – 7.16 (m, 5H), 6.81 – 6.52 (m, 2H), 6.29 – 5.91 (m, 4H), 4.74 – 4.11 (m, 14H), 4.08 (m, 1H), 3.99 – 3.81 (m, 3H), 3.80 – 3.51 (m, 14H), 3.41 (m, 1H), 3.35 (m, 5H), 3.29 (m, 2H), 3.13 (m, 1H), 3.08 – 2.89 (m, 5H), 2.85 – 2.71 (m, 1H), 2.51 (m, 2H), 2.40 – 2.18 (m, 2H), 2.17 – 1.66 (m, 6H), 1.66 – 1.52 (m, 1H), 1.51 – 1.21 (m, 3H), 1.21 – 0.76 (m, 24H). ^{13}C NMR (126 MHz, Methanol-d4) δ 174.03, 170.49, 169.11, 142.70, 135.48, 132.41, 128.94, 128.15, 127.86, 127.24, 127.06, 126.85, 126.71, 126.55, 85.27, 82.05, 77.48, 75.91, 70.35, 70.07, 70.00, 69.21, 68.75, 60.59, 60.18, 59.45, 59.19, 57.21, 56.93, 56.08, 55.26, 55.04, 54.74, 50.27, 49.91, 48.12, 48.06, 47.95, 47.89, 47.81, 47.78, 47.74, 47.72, 47.66, 47.60, 47.55, 47.43, 47.31, 47.26, 47.22, 47.09, 46.68, 44.74, 44.45, 44.12, 42.07, 34.47, 30.46, 29.00, 26.26, 25.59, 25.19, 24.46, 24.21, 23.06, 18.36, 17.76, 17.36, 15.59, 14.66, 14.55, 14.42, 13.71, 9.51. ESI-HRMS Calculated for $C_{59}H_{98}N_{11}O_{16}S_2$ $[M+H]^+$:1280.6634, Found:1280.6644. HPLC analysis: retention time = 5.067 min; peak area, 99%.

Compound 12S. Fluorescein isothiocyanate-N_3 (56 mg, 0.118 mmol), **5S** (49.192 mg, 0.142 mmol), Na-ascorbate (41.56 mg, 0.236 mmol) and $CuSO_4$ (37.54 mg, 0.236 mmol) were dissolved in tBuOH/H_2O/DMF (1/1/1, 6 mL). The reaction mixture was stirred at room temperature in the dark for 2 h. The resulting crude mixture was purified by HPLC to afford 43.1 mg (0.0524 mmol, 44.41% yield) **12S** as a yellow solid. ESI-HRMS Calculated for $C_{35}H_{35}N_8O_{10}S_3$ $[M+H]^+$:823.1638, Found:823.1646. ^{1}H NMR (500 MHz, DMSO-d6) δ 10.13 (s, 3H), 8.64 (t, *J* = 5.6 Hz, 1H), 8.20 (s, 1H), 8.13 (s, 1H), 7.92 (s, 1H), 7.70 (d, *J* = 8.1 Hz, 1H), 7.18 (d, *J* = 8.3 Hz, 1H), 6.76 (m, 2H), 6.67 (d, *J* = 2.0 Hz, 2H), 6.64 – 6.51 (m, 4H), 6.11 (m, 4H), 4.60 (t, *J* = 6.1 Hz, 2H), 4.42 (d, *J* = 1.9 Hz, 1H), 4.40 – 4.36 (m, 2H), 4.12 – 3.92 (m, 3H), 3.55 (d, *J* = 5.5 Hz, 2H), 3.47 (d, *J* = 11.6 Hz, 1H), 3.17 (d, *J* = 3.7 Hz, 1H), 2.91 (dd, *J* = 12.5, 4.2 Hz, 1H).

Compound 12R. Fluorescein isothiocyanate-N_3 (56 mg, 0.118 mmol), **5R** (49.192 mg, 0.142 mmol), Na-ascorbate (41.56 mg, 0.236 mmol) and $CuSO_4$ (37.54 mg, 0.236 mmol) were dissolved in tBuOH/H_2O/DMF (1/1/1, 6 mL). The reaction mixture was stirred at room temperature in the

dark for 2 h. The resulting crude mixture was purified by HPLC to afford 69.6 mg (0.0846 mmol, 71.68% yield) **12R** as a yellow solid. ESI-HRMS Calculated for $C_{35}H_{35}N_8O_{10}S_3$ $[M+H]^+$:823.1638, Found:823.1653. ^{1}H NMR (500 MHz, DMSO-d6) δ 10.13 (s, 3H), 8.63 (t, *J* = 5.6 Hz, 1H), 8.20 (s, 1H), 8.12 (d, *J* = 8.3 Hz, 1H), 7.93 (s, 1H), 7.71 (d, *J* = 8.1 Hz, 1H), 7.18 (t, *J* = 7.6 Hz, 1H), 6.82 – 6.70 (m, 2H), 6.67 (dd, *J* = 7.3, 2.3 Hz, 2H), 6.58 (qd, *J* = 8.7, 2.5 Hz, 4H), 6.20 – 5.97 (m, 4H), 4.61 (t, *J* = 6.1 Hz, 2H), 4.42 (d, *J* = 1.9 Hz, 1H), 4.40 – 4.36 (m, 2H), 3.97 (m, 3H), 3.55 (d, *J* = 5.5 Hz, 2H), 3.47 (d, *J* = 11.6 Hz, 1H), 3.17 (d, *J* = 2.7 Hz, 1H), 2.92 (dd, *J* = 12.5, 4.2 Hz, 1H).

4.3 Modification of peptide oxytocin with BVP linkers (5, 5R, 5S)

In a 330 µL 96 round well plates, peptide oxytocin (120 µL, 2.5 mM in phosphate buffer (pH = 7.4)) was reduced by addition of 1.5 equiv reducing agent tris(2-carboxyethyl)phosphine (14.4 µL, 25 mM stock solution in H_2O, pH 7.07 was adjusted by NaOH and H_3PO_4). The mixtures were reacted on Micro plate fast oscillator at room temperature for 1 hour to attain reduced oxytocin followed by 24 µL of BVP linkers (**5**, **5R** or **5S**) (25 mM in CH_3CN) and 41.6 µL of CH_3CN was added. The mixtures were reacted on Micro plate fast oscillator at room temperature for 2 hours. The resulting reaction mixture was analyzed by RP-HPLC (Supporting Information Figure S5). The product **6** was confirmed by LC-MS (Supporting Information Figure S6). Prepared using 100 µl of 6 solution (received from analytical HPLC), 20 µl of Ellman's reagent (25 mM in CH_3CN) and 20 µl of PBS (pH 10.02) at room temperature for 2 hours. The resulting mixture was analyzed by LC-MS. A 330 µL LABTIDE 96 Round Well contained 120 µL of peptide oxytocin (2.5 mM in phosphate buffer at pH = 7.4), 24 µL of BVP linkers (**5**, **5R**, **5S**) (25 mM in CH_3CN) and 56 µL of CH_3CN. The mixture was reacted on Micro plate fast oscillator at room temperature for 2 hours. The resulting reaction mixture was analyzed by RP-HPLC and LC-MS (Supporting Information Figure S7 and S8). No reaction occurred when oxytocin reacted with BVP linkers directly.

4.4 Antibody-Drug Conjugates

In a 330 µL 96 round well plates, trastuzumab (100 µL, 3 mg/mL in phosphate buffer (pH = 7.4)) was reduced by addition of fivefold molar excess reducing agent tris(2-carboxyethyl)phosphine (1 µL, 10 mM stock solution in H_2O, pH 7.07 was adjusted by NaOH and H_3PO_4). The mixtures were reacted on Micro plate fast oscillator at room temperature for 2 hours to attain reduced trastuzumab followed by 2 µL of **10**, **10R** or **10S** (10 mM stock solution in DMSO) was added, respectively. The mixtures were reacted on Micro plate fast oscillator at room temperature for 4

hours. The antibody-drug conjugates were purified using a Zeba™ Spin Desalting Columns (Thermo, 7K MWCO, 0.5 mL) to remove excess reagents and the resulting conjugates **11**, **11R** and **11S** were characterized by UPLC−MS analysis and Size-exclusion chromatography analysis (Supporting Information Figure S3).

4.5 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels

Non-reducing glycine-SDS-PAGE at 4-12% acrylamide gels were performed following standard lab procedures. A 4-12% stacking gel was used and a broad-range MW marker (10–250 kDa, Prestained PagerulerPlus Protein Standards, Bio-Rad) was co-run to estimate protein weights. Samples (10 µL at ~12 µM construct) were mixed with NuPAGE LDS sample buffer (4X, 10µL) and phosphate buffer ((pH = 7.4), 20 µL) were added to the tube. The solution (10 µL) was loaded to NuPAGE Bis-Tris mini gel with 4-12% gradient polyacrylamide concentration, and the conjugation reaction was analyzed by electrophoresis (120 V). The buffering system employed was 1X SDS running buffer (NuPAGE MOPS SDS running buffer, 20X, pH 7.3, 50 to 950 mL deionized water). After 50 min, the intensities of fluorescence were analyzed. Then, the gel was stained with coomassie dye (0.5%) and the gel was read 2 h after mixing at room temperature.

4.6 Papain digestion of trastuzumab and the conjugates 11, 11R and 11S

Trastuzumab (3 mg/mL) in digestion buffer was reacted with a 1/10 amount (wt./wt.) of immobilized papain (250 g/mL of gel) for 20 h at 37 °C under nitrogen in a buffer containing 20 mM sodium phosphate monobasic, 10 mM disodium EDTA and 80 mM Cys*HCl (pH 7.0). The Cys*HCl was incorporated immediately before trastuzumab digestion. After digestion, the mixture was centrifuged at 200 rcf for 5 min and the supernatant was removed for purification. The supernatant was concentrated to a volume of 200 L using a diafiltration column (30 KDa MWCO) to purify it from low-molecular-weight proteolytic contaminants, and buffer exchanged into phosphate-buffered saline (PBS, pH 7.0) by passage through diafiltration columns (30 KDa MWCO) four times with excessive PBS (pH 7.0). Finally, the sample was analysed by LC-MS and revealed a mixture of Fab products, LC-MS observed masses: 47270 and 47635. The conjugates **11**, **11R**, **11S** were also digested by using the protocol outlined.

4.7 Antibody-Fluorescence Conjugates

In a 330 µL 96 round well plates, trastuzumab solution in phosphate buffer (pH = 7.4) (3 mg/mL, 100 µL) and 4 µL of **12R** or **12S** (10 mM stock solution in DMSO) was mixed. The mixture was

reacted on Micro plate fast oscillator at room temperature in the dark for 12 hours. The antibody-fluorescence conjugates was purified using a Zeba™ Spin Desalting Columns (Thermo, 7K MWCO, 0.5 mL) to remove excess reagents and the resulting mixture was characterized by UPLC−MS analysis. In a 330 µL 96 round well plates, trastuzumab (100 µL, 3 mg/mL in phosphate buffer (pH = 7.4)) was reduced by addition of fivefold molar excess reducing agent tris(2-carboxyethyl)phosphine (1 µL, 10 mM stock solution in H_2O, pH 7.07 was adjusted by NaOH and H_3PO_4). The mixtures were reacted on Micro plate fast oscillator at room temperature for 2 hours to attain reduced trastuzumab followed by 4 µL of **12R** or **12S** (10 mM stock solution in DMSO) was added. The mixtures were reacted on Micro plate fast oscillator at room temperature in the dark for 12 hours. The antibody-fluorescence conjugates were purified using a Zeba™ Spin Desalting Columns (Thermo, 7K MWCO, 0.5 mL) to remove excess reagents and the resulting conjugates **13S** and **13R** were obtained.

4.8 The stability of the antibody-fluorescence conjugate 13S

In a 330 µL 96 round well plates, trastuzumab (300 µL, 3 mg/mL in phosphate buffer (pH = 7.4)) was reduced by addition of tenfold molar excess reducing agent tris(2-carboxyethyl)phosphine (2 µL, 10 mM stock solution in H_2O, pH 7.07 was adjusted by NaOH and H_3PO_4). The mixture was reacted on Micro plate fast oscillator at room temperature for 2 hours to attain reduced trastuzumab followed by 4 µL of **12S** (10 mM stock solution in DMSO) was added. The mixture was reacted on Micro plate fast oscillator at room temperature in the dark for 12 hours. The antibody-fluorescence conjugates **13S** was purified using a Zeba™ Spin Desalting Columns (Thermo, 7K MWCO, 0.5 mL) to remove excess reagents and divided into 2 parts, numbered as solution A and solution B, respectively.

Addition of 20 uL of human plasma to the solution A and the mixture was stored 37 ^{o}C in the dark. Addition of 33.8 uL of GSH (10 mM in water) to the solution B and the mixture was stored 37 ^{o}C in the dark. Aliquots at different incubation times were analyzed by SDS-PAGE. The frozen sample in the -80 ^{o}C refrigerator was taken out. NuPAGE LDS sample buffer (4X, 10µL) and NuPAGE reducing agent (100 mM, 1µL) were added to the tube. The solution was heated at 95 ^{o}C for 5 min. The heated solution (10 µL) was loaded to NuPAGE Bis-Tris mini gel with 4-12% gradient polyacrylamide concentration, and the conjugation reaction was analyzed by electrophoresis (120 V). (Supporting Information Figure S1)

4.9 The stability of the antibody-fluorescence conjugate 13R

In a 330 µL 96 round well plates, trastuzumab (300 µL, 3 mg/mL in phosphate buffer (pH = 7.4)) was reduced by addition of tenfold molar excess reducing agent tris(2-carboxyethyl)phosphine (2 µL, 10 mM stock solution in H_2O, pH 7.07 was adjusted by NaOH and H_3PO_4). The mixture was reacted on Micro plate fast oscillator at room temperature for 2 hours to attain reduced trastuzumab followed by 4 µL of **12R** (10 mM stock solution in DMSO) was added. The mixture was reacted on Micro plate fast oscillator at room temperature in the dark for 12 hours. The antibody-fluorescence conjugates **13R** was purified using a Zeba™ Spin Desalting Columns (Thermo, 7K MWCO, 0.5 mL) to remove excess reagents and divided into 2 parts, numbered as solution A and solution B, respectively. Addition of 20 uL of human plasma to the solution A and the mixture was stored 37 ^{o}C in the dark. Addition of 33.8 uL of GSH (10 mM in water) to the solution B and the mixture was stored 37 ^{o}C in the dark. Aliquots at different incubation times were analyzed by SDS-PAGE. The frozen sample in the -80 ^{o}C refrigerator was taken out. NuPAGE LDS sample buffer (4X, 10µL) and NuPAGE reducing agent (100 mM, 1µL) were added to the tube. The solution was heated at 95 ^{o}C for 5 min. The heated solution (10 µL) was loaded to NuPAGE Bis-Tris mini gel with 4-12% gradient polyacrylamide concentration, and the conjugation reaction was analyzed by electrophoresis (120 V) (Supporting Information Figure S2).

4.10 Cell lines and culture

Human breast cancer lines SK-BR-3, MCF7, MDA-MB-468, MDA-MB-231and human gastric cancer cell NCI-N87 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Fetal bovine serum (FBS), RPMI 1640 medium, DMEM medium, MEM medium, Penicillin-Streptomycin (PS), non-essential amino acid (NEAA) were purchased from Gibco Thermofisher Scientific (Waltham, MA. USA). Recombinant human insulin was purchased from Sigma Chemical (St. Louis, MO, USA). SK-BR-3, MDA-MB-468 and MDA-MB-231 cells were cultured in DMEM medium with 10% FBS and 1% PS; NCI-N87 cell was cultured in RPMI 1640 medium with 10% FBS and 1% PS; MCF7 cell was cultured in MEM medium with 10% FBS, 1% PS, 1% NEAA and 10 µg/mL insulin; all cells were cultured at 37 °C in a humidified incubator with 5% CO_2.[47]

4.11 Flow cytometry for affinity of ADCs

Goat Anti-Human IgG H&L (DyLight® 650) was purchased from Abcam (Cambridge, UK).

DAPI was purchased from Cell Signaling Technology (Boston, USA). SK-BR-3 and MCF7 cells (2×10^5 cells/tube) were collected and incubated with varying concentrations of ADCs and trastuzumab at 4 ^{o}C for 30 min. After being washed with FACE solution (1% BSA in PBS, pH 7.4), the cells were stained with Goat Anti-Human IgG H&L (DyLight® 650) (1:200) at 4 ^{o}C for 30 min. After being washed with FACE solution, the cells were stained with DAPI (1μg/mL) in PBS. The fluorescent signals in individual samples were analyzed by CytoFLEX flow cytometer (Beckman Coulter, Brea, USA) using the FlowJo software.

4.12 Flow cytometry for internalization of ADCs.

SK-BR-3 cell (2×10^5 cells/tube) was collected and incubated with ADCs and trastuzumab in duplicate at 4 ^{o}C for 30 min. After being washed with FACE solution, the samples were incubated with PBS at 4 ^{o}C and 37 ^{o}C for 30 min, 1 h, and 3 h, respectively. And then the samples were incubated with Goat Anti-Human IgG H&L (DyLight® 650) (1:200) at 4 ^{o}C for 30 min. After being washed with FACE solution, the cells were stained with DAPI (1μg/mL) in PBS. The fluorescent signals in individual samples were analyzed by CytoFLEX flow cytometer (Beckman Coulter, Brea, USA) using the FlowJo software. The percentage of internalization was determined using the following formula: percentage of internalization = (fluorescence intensity of cells at 4 ^{o}C – fluorescence intensity of cells at 37 ^{o}C)/ fluorescence intensity of cells at 4 ^{o}C × 100%.

4.13 Cytotoxicity assay

Cytotoxicity assay of ADCs in vitro was performed on SK-BR-3, NCI-N87, MCF7, MDA-MB-468 and MDA-MB-231 cells according to previous study (“Rossin R, Versteegen R M, Wu J, et al. Chemically triggered drug release from an antibody-drug conjugate leads to potent antitumour activity in mice. Nature communications, 2018, 9(1): 1484.”) with some modification. Briefly, cells (5×10^3 cells/well) were cultured in 96-well plates with 100 μL complete medium, and 24 h later the cells were treated in triplicate with varying concentrations (500 nM, 100 nM, 20 nM, 4 nM, 0.8 nM, 0.16nM, 32 pM, 6.4 pM, 1.28 pM and 0.256 pM) of ADCs, MMAE, T-DM1 and trastuzumab for 72 h. The cells cultured in medium alone served as the control and medium alone served as the blank. Cell viability was determined using Cell Counting Kit-8 (CCK-8) kit according to the manufacturer’s instructions. The absorbent optical density (OD) values at 450 nm were measured in a microplate reader (SpectraMax i3, MD, USA). The inhibition rate of cell growth in individual wells was determined using the following formula: growth inhibition rate =

(OD value of control – OD value of dose)/ (OD value of control – OD value of blank) × 100%. The half maximal inhibitory concentrations (IC_{50}) of the compounds for each cell were calculated using the Prism 7 software.

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Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/

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Bis(vinylsulfonyl)piperazines linkers enable selective disulfides re-bridging at Fab regions of antibody;

Formation of highly homogeneous ADCs with DAR of 2;

Declaration of Interest Statement

The authors declare no conflict of interest.