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Brief Communication

Selective Formation of Covalent Protein Heterodimers with an Unnatural Amino Acid

Benjamin M. Hutchins,[1,4] Stephanie A. Kazane,[1,4] Karin Staflin,[2] Jane S. Forsyth,[2] Brunhilde Felding-Habermann,[2] Vaughn V. Smider,[3,*] and Peter G. Schultz[1,*]
[1]Department of Chemistry and the Skaggs Institute for Chemical Biology
[2]Department of Molecular and Experimental Medicine
[3]Department of Molecular Biology
The Scripps Research Institute, La Jolla, CA 92037, USA
[4]These authors contributed equally to this work
*Correspondence: vvsmider@scripps.edu (V.V.S.), schultz@scripps.edu (P.G.S.)
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SUMMARY

We report a strategy for the generation of heterodimeric protein conjugates using an unnatural amino acid with orthogonal reactivity. This paper addresses the challenges of site-specificity and homogeneity with respect to the synthesis of bivalent proteins and antibody-drug conjugates. There are numerous antibody-drug conjugates in preclinical and clinical development, yet these are based either on nonspecific lysine coupling chemistry or on disulfide modification made difficult by the large number of cysteines in antibodies. Here, we describe a recombinant approach that can be used to rapidly generate a variety of constructs with defined conjugation sites. Moreover, this methodology results in homogeneous antibody conjugates whose biological, physical, and pharmacological properties can be quantitatively assessed and subsequently optimized. As proof of concept, we have generated anti-Her2 Fab-Saporin conjugates that demonstrate excellent potency in vitro.

INTRODUCTION

Antibodies coupled to other ''effector'' molecules are of considerable interest in oncology because of their ability to target tumor cells with high specificity while delivering a cytotoxic payload. Antibodies can be chemically coupled to potent natural or synthetic toxins, or in the case of bispecific antibodies, one arm can provide the binding function while the second arm can provide the effector function, often through recruitment of cytotoxic T cells (Yamaizumi et al., 1978). Although considerable effort is focused on the synthesis of effective antibody-drug conjugates (ADCs), and exciting clinical results have been obtained with trastuzumab-DM1 and brentuximab vedotin (SGN-35) for breast cancer and Hodgkin lymphoma, respectively, there still remain major challenges in the field (Presta, 2006; Senter, 2009). Even though there are a large number of antibody drug conjugates in preclinical and clinical development, only three conjugated antibodies have been approved by the Food and Drug Administration (FDA): anti-CD20-^{90}Y (Ibritumomab) and antiCD20 ^{131}I (Tositumomab) both for non-Hodgkin lymphoma, and anti-CD33-calicheamicin (Gemtuzumab Ozogamicin) for acute myelogenous leukemia (Wu and Senter, 2005). Despite the promise of these ADCs, Gemtuzumab Ozogamicin was recently pulled from the market due to inactivity and toxicity (Beck et al., 2010; Bross et al., 2001; Hamann et al., 2002; Hollander et al., 2008; Senter, 2009). In general, coupling of the toxin by amide bond formation with lysine side chains results in nonspecific conjugation which can alter antigen binding, physical properties, biological activity, or pharmacokinetics (Wu and Senter, 2005; Junutula et al., 2008). Moreover, the inability to even define the structure and composition of randomly labeled proteins (in contrast to the homogenous nature of synthetic drugs) has been a significant challenge in optimizing drug efficacy, safety, and manufacturing. There are some cases where lysine free variants of proteins are engineered to alleviate this problem; however this cannot be done without complicated engineering (removal of multiple lysines) which can alter protein binding and/or activity (Bachran et al., 2011). Cysteine labeling is largely restricted to proteins without additional reactive cysteines in order to prevent unwanted disulfide bridge formation, or aggregation. If this requirement is not met, as can be the case with antibodies, more complex labeling protocols to avoid reaction with native cysteines must be performed, which can limit the sites of conjugation, stability of conjugates or lead to challenges in scale up (Schibli et al., 2002; Junutula et al., 2008).

Bispecific antibodies have recently been shown to have very promising therapeutic activity, yet in most cases, multivalency is limited to either naturally bivalent IgGs or fusion proteins (Wolf et al., 2005). Fusion protein constructs require end-to-end connection of two antibody coding regions; however, the spatial proximity of the N termini of the variable heavy chain (V_H) and the variable light chain (V_L) to the antigen binding site may potentially affect antigen binding (Verma et al., 1998). The production of bispecific antibodies can also pose significant challenges as they are often inactive, or form unwanted aggregates that may affect production and safety (Woo et al., 2006; Zhou et al., 2007).

The availability of additional genetically encoded amino acids with unique chemical reactivity may provide a solution to many of these challenges. Previously we have site-specifically

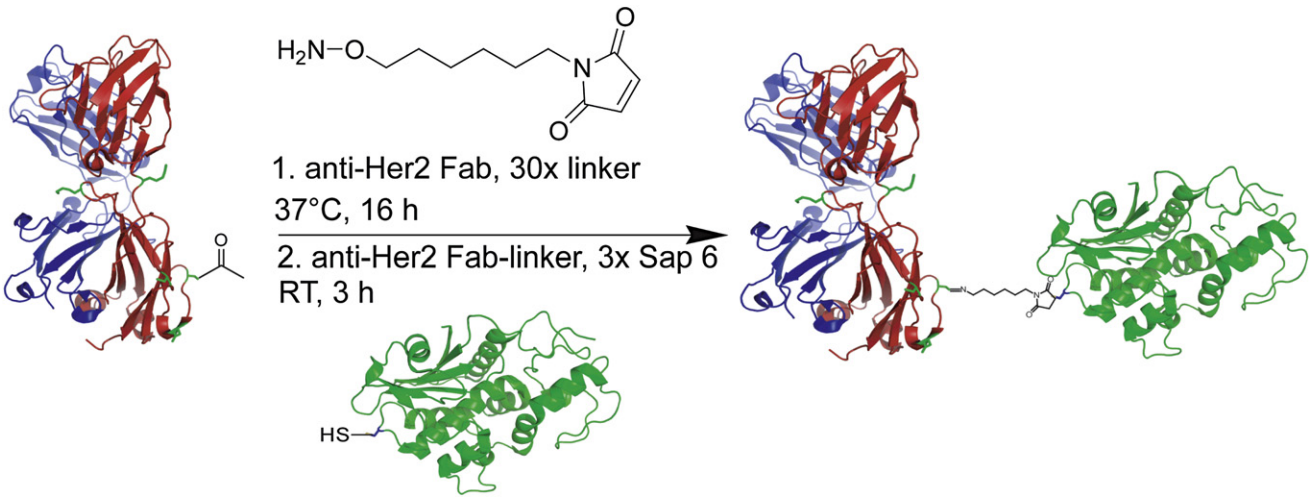

Figure 1. Synthesis of a Site-Specific Anti-Her2 Fab-Sap 6 Heterodimer
An excess of aminooxy-maleimide linker is added to pAcPhe Herceptin Fab (blue/red). After purification, the toxin Sap 6 A157C (green) is added to the maleimide functionalized Fab to create the anti-Her2-Sap heterodimer. See also Figure S1.

incorporated *p*-acetylphenylalanine (pAcPhe) and *p*-azidophenylalanine into proteins in response to amber nonsense codons in bacteria and mammalian cells (Wang et al., 2003, 2006; Hutchins et al., 2011; Chin et al., 2002; Young et al., 2010). These amino acids have orthogonal chemical reactivity to the canonical twenty amino acids and can be conjugated efficiently and selectively to alkoxyamines or alkynes, respectively, under mild conditions. The site-specific introduction of a keto or azide group into a surface exposed site in an antibody should allow the selective conjugation of other proteins (antibodies, natural toxins, etc.) or synthetic reagents (toxins, oligonucleotides, metal chelators, etc.), while avoiding reaction with the native disulfide bonds which are required for correct folding. Moreover, the mutant proteins can be produced by recombinant methods rapidly and on a large scale. As a proof of concept, we now report the synthesis of a site-specific heterodimeric antibody-toxin conjugate between the protein toxin saporin and Herceptin (trastuzumab, anti-Her2) Fab that possesses high potency in vitro.

RESULTS AND DISCUSSION

Mutant anti-Her2 Fab and Saporin Expression

Herceptin is an approved monoclonal antibody for the treatment of Her2 overexpressing breast cancers (Ross et al., 2009). pAcPhe was site-specifically introduced into Herceptin Fab using an orthogonal amber suppressor aminoacyl-tRNA synthetase/tRNA pair specific for pAcPhe. The previously reported pBAD/pEVOL expression system was used to substitute pAcPhe at a surface exposed site (K169 or S202) (Hutchins et al., 2011; Young et al., 2010) and both have been shown to have excellent coupling efficiencies with aminooxy modified AlexaFluor 488 dye (Hutchins et al., 2011). Both the anti-Her2 K169pAcPhe and anti-Her2 S202pAcPhe mutant Fabs were expressed in *Escherichia coli* in good yields (>1 mg/liter in shake flasks), purified by Protein G chromatography, and their masses confirmed by electrospray ionization mass spectrometry (ESI-MS) (see Figures S1A–S1B available online).

Saporin was used as the toxin partner as it is a highly cytotoxic non-cell-permeable enzyme that functions as a ribosome-inactivating protein (RIP) and has been used as an effector in targeting EGFR positive cells (Chandler et al., 1998). A type 1 RIP, Saporin 6 (Sap 6), has been shown to have a genomic DNA fragmentation activity in addition to its RNA N-glycosidase activity (Bagga et al., 2003). Since Sap 6 does not contain any cysteines, we were able to mutate an alanine to a cysteine residue in wild-type Saporin to generate a uniquely reactive site for conjugation (pET-A157C). When compared with wild-type expression in *E. coli* shake flasks, we were able to obtain similar yields from the cysteine mutant (1 mg/liter) (Pittaluga et al., 2005; Bonini et al., 2006). The protein was purified by cation exchange (Mono S 5/50 GL) and size exclusion chromatography (Superdex 75 10/300 GL). The expected molecular weight (29 kDa) was confirmed by SDS-page gel and ESI-MS (see Figures S1C and S1D).

Site-Specific Conjugation

To site-specifically crosslink the mutant Fab to saporin, we synthesized a bifunctional aminooxy-maleimide linker that can be selectively coupled to both the keto group of pAcPhe in anti-Her2 and the thiol group of cysteine in Sap 6 (see Figure 1). The desired compound was synthesized from commercial 6-bromohexanol in five steps (see Supplemental Experimental Procedures) (Defrancq and Lhomme, 2001; Toyokuni et al., 2003; Berndt et al., 2007). The aminooxy-maleimide linker (30 equiv.) was added to anti-Her2 K169pAcPhe Fab (100 μM) in acetate buffer (pH 4.5) and after 16 hr, the conjugate was confirmed by ESI-MS (see Figure 1E). It was then purified by size exclusion chromatography (Superdex 75 10/300) and three equivalents (240 μM) of Sap 6 A157C (reduced with TCEP resin) were added for 3 hr at room temperature. The heterodimer was purified using size exclusion chromatography (Superdex 200 10/300 GL) (see Figures S2B–S2D). The coupling efficiency of

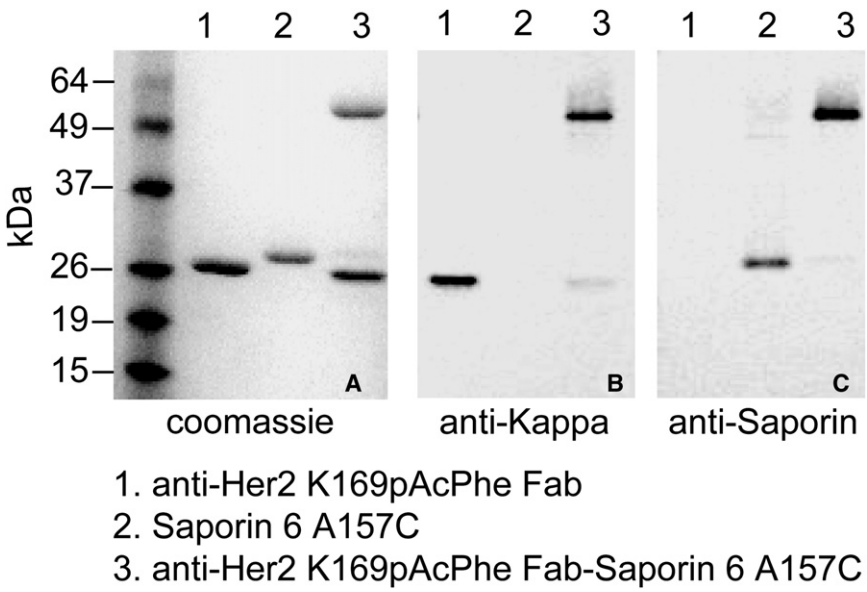

Figure 2. Characterization of Anti-HER2 K169pAcPhe Fab-Sap 6 A157C Heterodimeric Construct

Anti-Her2 K169pAcPhe Fab (lane 1) was conjugated to Sap 6 A157C (lane 2) as described in Figure 1 and the purified construct was analyzed by reducing SDS-page and stained with Coomassie (lane 3, A). The proteins were also transferred to a nitrocellulose membrane and incubated with anti-kappa-HRP (B) or anti-saporin-HRP (C). See also Figure S2.

anti-Her2 K169pAcPhe to Sap 6 A157C was ~50% (see Figure S2A). To verify the conjugation of Sap 6 to anti-Her2 Fab, we performed a western blot with both anti-kappa (Figure 2B) and anti-saporin antibodies (Figure 2C). Anti-Her2 Fab was reduced to the light and heavy chains at the appropriate molecular weight (25 kD) and the Her2 Fab-saporin construct was also partially reduced. As depicted in the SDS-page gel in Figure 2, lane 3, Saporin is correctly conjugated to the light chain of the anti-Her2-Fab producing a molecule of 55 kDa. The molecular weight of the heterodimer (77 kDa) was also confirmed by ESI-MS (see Figure S1F). The versatile nature of this system allowed us to easily generate another bivalent molecule using a different mutant site in the herceptin Fab (anti-Her2 S202pAcPhe) with a similar overall yield.

Cytotoxicity of anti-Her2 Fab-Saporin Heterodimer

We next tested the cytotoxicity of these heterodimeric conjugates with different human breast cancer cell lines. Anti-Her2 wild-type Fab and unconjugated Sap 6 A157C served as controls. MDA-MB-435 cells (Chambers, 2009; Hollestelle and Schutte, 2009) were transduced to express Her2 using a lentiviral system; cells receiving an empty vector served as the control (see Supplemental Experimental Procedures). Anti-Her2 conjugates or unconjugated proteins were added to cells at a concentration range of 2.7 pM to 20 nM, and cell viability (OD_{405}) was measured after 4 days. As shown in Figure 3, both the anti-Her2 wild-type Fab and Saporin 6 A157C have minimal, if any, effect on either Her2 positive or Her2 negative cells. There is only a minimal effect on cell viability by Saporin at the highest concentration (20 nM). However, when Saporin is conjugated to either anti-Her2 K169pAcPhe Fab or anti-Her2 S202pAcPhe Fab a dramatic increase in the concentration dependent cytotoxicity was observed and the corresponding EC_{50}'s were determined (197 and 154 pM, respectively). In the Her2 positive cell line, there is almost complete cell death at >1 nM concentrations, while the majority of the cells are still viable in the Her2 negative cell line under similar conditions. The EC_{50}'s of both unconjugated anti-Her2 Fab and Saporin in both the positive and negative cells lines are greater than the highest concentration used (>20 nM). Similar EC_{50}'s were determined for the anti-Her2-Sap conjugates in the Her2 negative cell line. In addition to these cell lines, other lines (SKBR3, BCM2) have been used and similar results were obtained (see Figure S3). Thus, cytotoxicity is specific to Her2 expressing cells, and requires both components of the Her2-Saporin conjugate.

SIGNIFICANCE

We have demonstrated that bivalent anti-Her2-Saporin conjugates are very effective *in vitro* in inducing cell death in Her2 positive breast cancer cells. We were also able to combine the specificity of antibodies and the potency of protein toxins using a bioorthogonal chemistry that produced homogeneous heterodimeric conjugates in good yields. These constructs and the resulting full-length antibody conjugates will next be tested in rodent xenograft models. Using unnatural amino acid chemistry, we can control the site of conjugation, create highly stable homogeneous conjugates, and combine the orthogonal reactivities of the cysteine and pAcPhe moieties to create even more complex conjugates. Moreover, with this recombinant-based approach one can rapidly create numerous heterodimeric constructs with different toxins and antibodies to determine the optimal conjugation partner, site, and linker to create the most effective therapeutic agents.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

To express Saporin 6 A157C, plasmid pET-SapA157C (which encodes a Saporin 6 gene with a cysteine mutant and an ampicillin-resistant gene) was transformed into BL21(DE3) pLysS *E. coli*. Cells were amplified in 2YT media (5 ml) supplemented with ampicillin (50 μg/ml) and chloramphenicol (40 μg/ml). Cells were grown to saturation and inoculated 1:100 in 2YT expression media at 37°C (250–270 rpm shaker). At an OD of 0.6–0.8, the cells were induced with 1 mM IPTG and continued shaking at 37°C for 4 hr. The cells were centrifuged (6000 × g; 10 min) and resuspended in 40 ml lysis buffer (20 mM Tris, [pH 8.0], 2 mM EDTA, complete protease inhibitor tablet (Roche), and 1–5 mM DTT) per 1 liter culture. The sample was sonicated on ice (10 min process time, 30 s on, 30 s off) and centrifuged at 30,000 × g for 20 min.

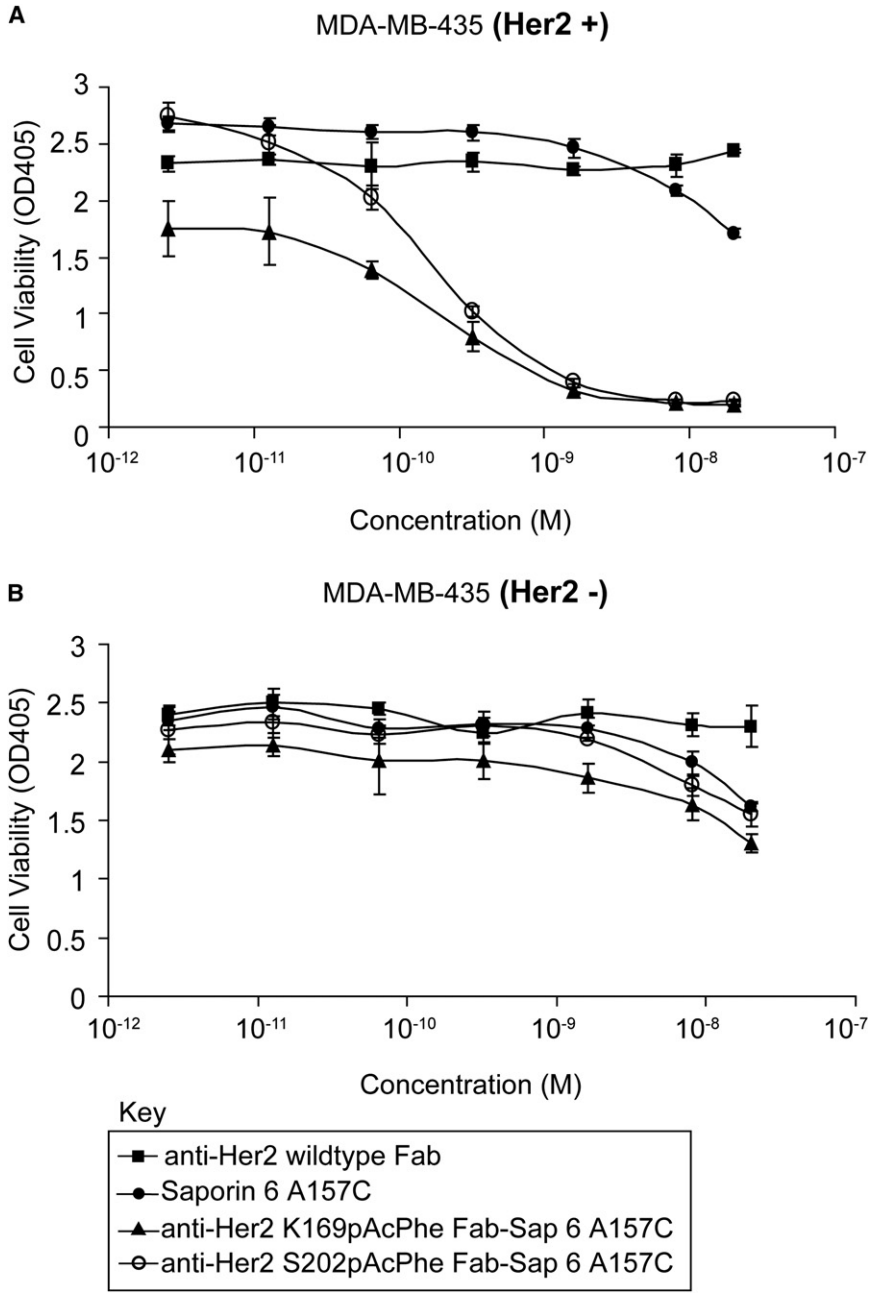

Figure 3. Cell Viability Assay with Anti-Her2 pAcPhe Fab-Sap 6 A157C Bivalent Constructs

Anti-Her2 Fab-Sap heterodimeric constructs and controls were incubated with MDA-MB-435 cells and the OD_{405} (*p*-nitrophenyl phosphate, pNPP) was read after 4 days. Concentrations of samples range from 2.7 pM to 20 nM.

(A) Her2 positive cell line.

(B) Her2 negative cell line.

See also Figure S3.

Saporin was purified by cation exchange chromatography (Mono S 5/50 GL), concentrated by Amicon concentrator (10 kDa MWCO), 1 mM dithiothreitol (DTT) was added, and sample was purified by size exclusion chromatography (Superdex 75 10/300 GL). Anti-Her2 Fabs were expressed and purified as described previously (Hutchins et al., 2011).

Site-Specific Conjugation

Fab (100 μM) (anti-Her2 K169pAcPhe) was reacted with 3 mM crosslinker in 100 mM acetate buffer (pH 4.5) at 37°C. After 16 hr, the conjugate was purified by size exclusion chromatography (Superdex 75 10/300 GL). Sap-6 (A157C) was then reduced using TCEP resin (2 h) and 240 μM protein was added to 80 μM Fab-linker in PBS (pH 7.5) for 3 hr at room temperature. The coupled protein was purified using size exclusion chromatography (Superdex 200 10/300 GL) and resolved on reducing SDS-page and stained with Coomassie (Figure 2, a), or transferred to nitrocellulose and probed with anti-kappa-HRP (Sigma, 1:5000 dilution) or anti-saporin-HRP (Advanced Targeting Systems, 1:2000 dilution) in 3% dry milk (BioRad) in PBST (0.05% Tween). Membranes were developed colorimetrically using the metal enhanced DAB kit (Pierce).

Cell Viability Assay

Human tumor cells were seeded into 96-well plates at 5000 cells/well in complete EMEM medium containing 10% FBS. The cells were allowed to attach for 2 hr at 37°C before adding Her2 Fab conjugates or controls at a concentration range from 0 to 20 nM. After 4 days, the cells were washed and lysed with 1% Triton X-100. The number of live cells was determined based on phosphatase activity measured photometrically after incubation with p-nitrophenyl phosphate (pNPP, Sigma).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at doi:10.1016/j.chembiol.2011.01.006.

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