Bioconjugate Chemistry

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Transglutaminase-Based Chemo-Enzymatic Conjugation Approach Yields Homogeneous Antibody–Drug Conjugates

Patrick Dennler,[†] Aristeidis Chiotellis,[‡] Eliane Fischer,[†] Delphine Brégeon,[§] Christian Belmant,[§] Laurent Gauthier,[§] Florence Lhospice,[§] François Romagne,[§] and Roger Schibli[†,*,‡]

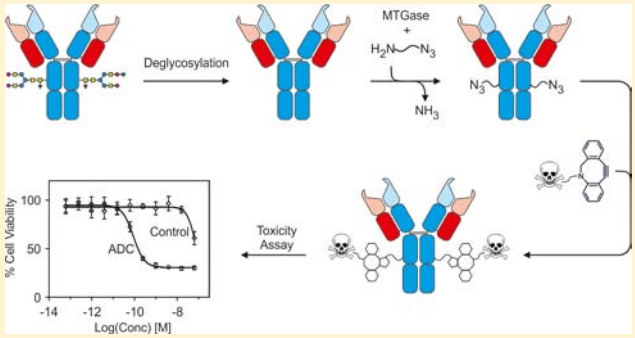
[†]Center for Radiopharmaceutical Sciences ETH-PSI-USZ, Paul Scherrer Institute, 5232 Villigen PSI, Switzerland

[‡]Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences, ETH Zurich, 8092 Zurich, Switzerland

[§]Innate-Pharma SA, 13276 Marseille, France

Supporting Information

ABSTRACT: Most chemical techniques used to produce antibody–drug conjugates (ADCs) result in a heterogeneous mixture of species with variable drug-to-antibody ratios (DAR) which will potentially display different pharmacokinetics, stability, and safety profiles. Here we investigated two strategies to obtain homogeneous ADCs based on site-specific modification of deglycosylated antibodies by microbial transglutaminase (MTGase), which forms isopeptidic bonds between Gln and Lys residues. We have previously shown that MTGase solely recognizes Gln295 within the heavy chain of IgGs as a substrate and can therefore be exploited to generate ADCs with an exact DAR of 2. The first strategy included the direct, one-step attachment of the antimitotic toxin monomethyl auristatin E (MMAE) to the antibody via different spacer entities with a primary amine functionality that is recognized as a substrate by MTGase. The second strategy was a chemo-enzymatic, two-step approach whereby a reactive spacer entity comprising a bio-orthogonal thiol or azide function was attached to the antibody by MTGase and subsequently reacted with a suitable MMAE-derivative. To this aim, we investigated two different chemical approaches, namely, thiol-maleimide and strain-promoted azide–alkyne cycloaddition (SPAAC). Direct enzymatic attachment of MMAE-spacer derivatives at an 80 molar excess of drug yielded heterogeneous ADCs with a DAR of between 1.0 to 1.6. In contrast to this, the chemo-enzymatic approach only required a 2.5 molar excess of toxin to yield homogeneous ADCs with a DAR of 2.0 in the case of SPAAC and 1.8 for the thiol-maleimide approach. As a proof-of-concept, trastuzumab (Herceptin) was armed with the MMAE via the chemo-enzymatic approach using SPAAC and tested *in vitro*. Trastuzumab-MMAE efficiently killed BT-474 and SK-BR-3 cells with an IC_{50} of 89.0 pM and 21.7 pM, respectively. Thus, the chemo-enzymatic approach using MTGase is an elegant strategy to form ADCs with a defined DAR of 2. Furthermore, the approach is directly applicable to a broad variety of antibodies as it does not require prior genetic modifications of the antibody sequence.



INTRODUCTION

Antibody–drug conjugates (ADCs) are promising therapeutics since their tumor selectivity is increased when compared to classical chemotherapy or combination therapy.[1−6] Targeted delivery of toxic payload to the tumor leads to a potential reduction of off-target toxicity and therefore an increased therapeutic index.

Antibody conjugation methods typically include chemical reaction of lysines or cysteines with activated esters or maleimide functionality respectively. However, these reactions are difficult to control with regard to site-specificity and stoichiometry, which leads to heterogeneous products.[7] Moreover, each subpopulation of antibody conjugates produced in this manner will have different pharmacokinetic, efficacy, and safety profiles.[8,9]

Various technologies have been developed in recent years to improve the production of homogeneous ADCs. Junutula et al. endowed antibodies with additional cysteines that can be reacted with maleimide-functionalized toxins, thereby yielding nearly homogeneous conjugates with improved therapeutic indexes.[10] However, this method includes a reduction-reoxidation step that can potentially lead to unpaired, reactive sulfhydryl groups. Furthermore, depending on the location of the engineered cysteines, maleimide exchange processes can lead to drug loss and thus decrease the potency of the ADC.[11] Incorporation of non-natural amino acids into the backbone of an antibody represents another promising technology toward homogeneous ADCs. To this aim, *p*-acetylphenylalanine was introduced into an antibody, thereby creating a bioorthogonal functionality that was exploited for site-specific modification

Received: December 22, 2013
Revised: January 31, 2014
Published: February 3, 2014

with a drug.[12,13] Although this is an elegant approach, it is a rather complex process and has yet to prove its general applicability.

By targeting the carbohydrate moiety of an antibody, Zuberbühler et al. established a method to generate uniform ADCs while using native antibodies. A selective periodate oxidation of the fucose residue yielded an aldehyde functionality that could then be selectively reacted with a hydrazide-functionalized dolastatin analogue.[14] A major drawback of this approach is the huge excess (100-fold molar excess) of toxin that is required for the reaction since toxins are expensive and may cause hazardous waste during the manufacturing procedure. Okeley et al. also targeted the carbohydrate moiety as a potential site to attach drugs. In contrast to Zuberbühler et al., they introduced unnatural sugars by producing their antibodies by CHO cells in the presence of 6-thiofucose and subsequently conjugated the toxin to the antibody by using thiol-maleimide chemistry. Even though the excess of toxin could be reduced to a minimum here, incomplete incorporation of 6-thiofucose (60−70%) resulted in heterogeneous ADC product.[15]

Enzymatic modification of antibodies and other proteins represents an alternative approach to the chemical modification strategies outlined above.[16] Transglutaminases (TGase) are a family of enzymes (EC 2.3.2.13) that catalyze the formation of a covalent bond between the γ-carbonyl amide group of glutamines and the primary amine of lysines (Figure 1).[17] Since

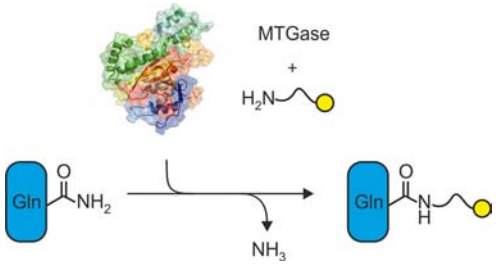


Figure 1. Microbial transglutaminase (MTGase) catalyzes the formation of a covalent bond between primary amines and the γ-carbonylamid group of glutamines under the loss of ammonia. (Structure from RCSB Protein Data Bank, 11U4.)

some TGases also accept substrates other than lysine as the amine donor, they have been used to modify proteins[18−20] including antibodies.[21−24] Recently, Strop et al. investigated the conjugation of drugs to antibodies using microbial transglutaminase (MTGase). By genetically introducing glutamine tags, i.e., a minimum of 4 amino acids, at various positions in an antibody they could demonstrate the impact of conjugation sites on the drug-to-antibody ratio (DAR), stability, and pharmacokinetics of ADCs.[25]

Here we report a procedure to obtain homogeneous ADCs that does not require prior engineering of the immunoglobulin at the DNA level. Our group has previously demonstrated that a conserved glutamine located in the constant domain of the heavy chain is the sole γ-carbonyl amide donor for MTGase within the backbone of a deglycosylated IgG1.[21] Small molecular weight substrates including cadaverin-derivatized chelators or biotin were efficiently coupled to this site.[26] We adapted this strategy to generate ADCs with a known site of conjugation and uniform stoichiometry. Furthermore, we investigated and compared two different conjugation strategies with regard to the amount of toxin that is required and the reaction efficiency (Figure 2). In addition to the direct enzymatic coupling of the toxin to the antibody, we established a novel chemo-enzymatic approach whereby a small linker with a synthetic functional group was first attached to the antibody by MTGase. A toxin was then chemically conjugated to the antibody to generate the desired ADC. Finally, the toxicity of the novel conjugate was evaluated *in vitro*.

EXPERIMENTAL PROCEDURES

Chemistry. All solvents used for reactions were purchased as anhydrous grade from Acros Organics (puriss., dried over molecular sieves, H_2O < 0.005%) and were used without further purification unless otherwise stated. Solvents for extractions, column chromatography, and thin layer chromatography (TLC) were purchased as commercial grade. All nonaqueous reactions were performed under an argon atmosphere using flame-dried glassware and standard syringe/septa techniques. Commercially available reagents were used without further purification. In general, reactions were magnetically stirred and monitored by TLC performed on Merck TLC glass sheets (silica gel 60 F254). Spots were visualized with UV light (λ = 254 nm) or by staining with either anisaldehyde solution or $KMnO_4$ solution and subsequent heating. Chromatographic purification of products was performed using Fluka silica gel 60 for preparative column chromatography.

11-Azido-3,6,9-trioxaundecan-1-amine (Azido-PEG3-Amine) and dibenzylcyclooctyne-amine (DBCO-amine) were purchased from Click Chemistry Tools (Scottsdale, USA). Amine-PEG4-vc-PAB-MMAE, DBCO-PEG4-vc-PAB-MMAE, and maleimide-vc-PAB-MMAE were purchased from ADC Biotechnology (St Asaph, UK), maleimide-vc-PAB-MMAF was purchased from Concortis (San Diego, USA). All of the toxins were equipped with a valine-citrulline 4-aminobenzyl alcohol (vc-PAB) cleavable linker unit.

Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ or CD_3OD on a Bruker Av-400 spectrometer at room temperature (RT). The measured chemical shifts are reported in δ (ppm) and the residual signal of the solvent was used as the internal standard ($CDCl_3$ ^1H: δ = 7.26 ppm, ^{13}C: δ = 77.0 ppm, CD_3OD ^1H: δ = 3.31 ppm, ^{13}C: δ = 49.1 ppm). All ^{13}C NMR spectra were measured with complete proton decoupling. Data of NMR spectra are reported as follows: s = singlet, d = doublet, t = triplet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets, br = broad signal. The coupling constant *J* is reported in Hertz (Hz). High resolution mass spectra (HR-MS) were recorded on a Waters Micromass Autospec Ultima (EI-sector) or a Varian Ionspec Ultima (MALDI/ESI-FT-ICR) (both MS service of Laboratory of Organic Chemistry (LOC) at the ETH Zurich). Low resolution mass spectra (LR-MS) were obtained with a Micromass Quattro micro API LC-ESI instrument.

HPLC was performed on a Merck-Hitachi L-7000 system equipped with a L-7400 tunable absorption detector. Analytical HPLC was performed with a reverse phase column (Ultimate XB C-18, 4.6 × 250 mm, 5 μm, Welch Materials Inc.) using 50 mM NH_4HCO_3 solution (solvent A) and acetonitrile (solvent B) with the following gradient system: 0−22 min, 5−80% B; 22−25 min, 80% B; 25−27 min, 80−5% B; 27−30 min, 5% B; flow, 1 mL/min; UV, 254 nm. Semipreparative HPLC purifications were performed with a reverse phase semi-preparative column (Xbridge C18, 10 × 150 mm, 5 μm,

dx.doi.org/10.1021/bc400574z | *Bioconjugate Chem.* 2014, 25, 569−578

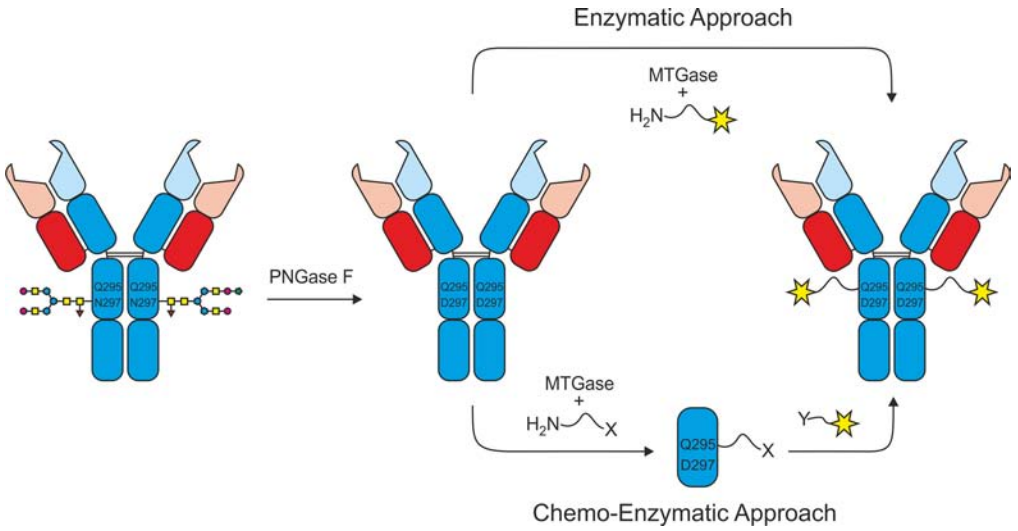


Figure 2. Schematic illustration of the two different strategies to generate homogeneous antibody-drug conjugates (ADCs). The N-linked glycans are cleaved by Peptide-N-Glycosidase F (PNGase F). The enzymatic approach includes direct conjugation of an amine-functionalized toxin (yellow star, top panel) to the deglycosylated antibody. For the chemo-enzymatic approach, a bioorthogonal functionality is enzymatically attached to a deglycosylated antibody (amine-X, bottom panel) in a first reaction. In a second step, the toxin (Y-yellow star), which is modified with a suitable functionality Y, is conjugated to the antibody.

Waters) with 50 mM NH_4HCO_3 solution (solvent A) and acetonitrile (solvent B) with the following gradient systems: system 1: 0−5 min, 40% B; 5−20 min, 40−80% B; flow, 4 mL/min; UV, 254 nM; system 2: 0−5 min, 30% B; 5−20 min, 30−70% B; flow rate, 4 mL/min; UV, 254 nm.

Di-*tert*-butyl(((6,6′-disulfanediylbis(hexanoyl))bis(azanediyl))bis(pentane-5,1-diyl))dicarbamate (1). In a solution of 6,6′-disulfanediyldihexanoic acid (250 mg, 0.849 mmol), *tert*-butyl (5-amino-pentyl)carbamate (412 mg, 2.038 mmol), and DIPEA (0.890 mL, 5.09 mmol) in DMF (4.7 mL), HBTU (1.29 g, 3.40 mmol) was added portionwise at RT. After stirring for 20 h, the yellowish reaction mixture was diluted with ethyl acetate (70 mL) and washed with cold HCl 0.1 N (3 × 50 mL), $NaHCO_3$ (sat) (1 × 50 mL), water (1 × 50 mL), and brine (1 × 50 mL). The organic layer was dried over sodium sulfate, filtered, and evaporated to dryness. The crude was purified by flash column chromatography on silica using $CHCl_3$/EtOH 95:5 to yield 525 mg (93%) of compound as a yellow sticky solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.87 (br, 2 H), 4.64 (br, 2 H), 3.22 (dt, J_1 = 7.3 Hz, J_2 = 6.8 Hz, 4H), 3.09 (dt, J_1 = 8.1 Hz, J_2 = 6.7 Hz, 4H), 2.65 (t, J = 7.2 Hz, 4H), 2.16 (t, J = 7.2 Hz, 4H), 1.73 − 1.59 (m, 8H), 1.55 − 1.45 (m, 8H), 1.42 (s, 18H), 1.37 − 1.28 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.9, 156.1, 79.0, 40.2, 39.2, 38.8, 36.5, 29.7, 29.1, 28.8, 28.4, 28.0, 25.3, 23.9. ESI-QTOF MS *m*/*z* calculated for $C_{32}H_{62}N_4O_6S_2$ $[M+H]^+$ 663.4184, measured 663.4185.

***tert*-Butyl (5-(6-Mercaptohexanamido)pentyl)-carbamate (2).** To a solution of di-*tert*-butyl(((6,6′-disulfanediylbis(hexanoyl))bis(azanediyl))bis(pentane-5,1-diyl))dicarbamate (196 mg, 0.296 mmol) in a mixture of tetrahydrofurane (3 mL) and water (0.31 mL, 17.21 mmol), tributylphosphine (272 μL, 1.035 mmol) was added dropwise at RT, within 1 min. The reaction mixture was stirred for 1 h and then the volatiles were removed under reduced pressure at 33 °C. The crude was azeotroped once with 50 mL benzene to remove traces of water and the residue was purified with flash column chromatography on silica with chloroform/ethanol 95:5 to yield a slightly yellow clear oil. NMR revealed that the compound was contaminated with oxidized tributylphosphine byproducts so the crude was purified again by flash column chromatography with hexane/ethyl acetate 2:8 to yield 180 mg (91%) of product as a colorless oil which solidified after storage at −25 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.88 (br, 1 H), 4.57 (br, 1 H), 3.23 (dt, J_1 = 7.3 Hz, J_2 = 6.9 Hz, 2H), 3.09 (dt, J_1 = 7.8 Hz, J_2 = 6.5 Hz, 2H), 2.52 (dt, J_1 = 8.0 Hz, J_2 = 7.6 Hz, 2H), 2.16 (t, J = 7.5 Hz, 4H), 1.69 − 1.57 (m, 4H), 1.56 − 1.46 (m, 4H), 1.43 (s, 9H), 1.36 − 1.28 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.8, 156.1, 79.1, 40.2, 39.2, 36.5, 33.6, 29.7, 29.1, 28.4, 27.9, 25.1, 24.4, 23.9. ESI-QTOF MS *m*/*z* calculated for $C_{16}H_{32}N_2O_3S$ $[M+H]^+$ 333.2206, measured 333.2198.

***S*-(6-((5-((*tert*-Butoxycarbonyl)amino)pentyl)amino)-6-oxohexyl) ethanethioate (3).** To a solution of *tert*-butyl (5-(6-mercaptohexanamido)pentyl)carbamate (180 mg, 0.541 mmol) and dry potassium carbonate (150 mg, 1.083 mmol) in degassed (freeze−pump−thaw) ethyl acetate (2.2 mL), acetic anhydride (61 μL, 0.650 mmol) was added and the reaction was stirred for 16 h. The reaction was then diluted with ethyl acetate (20 mL), filtered, and washed with cold water (1 × 10 mL) and brine (1 × 10 mL), dried over sodium sulfate, and evaporated to dryness. The crude was purified by flash column chromatography using chloroform/ethanol 96:4 to yield 182 mg (90%) of a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.68 (br, 1 H), 4.61 (br, 1 H), 3.21 (dt, J_1 = 7.3 Hz, J_2 = 6.9 Hz, 2H), 3.09 (dt, J_1 = 7.7 Hz, J_2 = 6.4 Hz, 2H), 2.83 (t, J = 7.2 Hz, 2H), 2.30 (s, 1H), 2.14 (t, J = 7.2 Hz, 2H), 1.67 − 1.44 (m, 8H), 1.42 (s, 9H), 1.40 − 1.27 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 196.0, 172.8, 156.1, 79.3, 40.2, 39.2, 36.4, 30.6, 29.7, 29.2, 29.1, 28.8, 28.4, 28.3, 25.1, 23.9. ESI-QTOF MS *m*/*z* calculated for $C_{18}H_{34}N_2O_4S$ $[M+H]^+$ 375.2312, measured 375.2312.

***S*-(6-((5-Aminopentyl)amino)-6-oxohexyl) ethanethioate (4, C6-SAc linker).** To a solution of *S*-(6-((5-((*tert*-butoxycarbonyl)amino)pentyl)amino)-6-oxohexyl) ethanethioate (187 mg, 0.5 mmol) in dichloromethane (6.6 mL), trifluoroacetic acid (0.77 mL, 5.34 mmol) was added dropwise at 0 °C. After stirring for 10 min, the reaction mixture was allowed to reach RT where it was stirred for 1 h. The volatiles were removed under reduced pressure at 30 °C and the residue

dx.doi.org/10.1021/bc400574z | *Bioconjugate Chem.* 2014, 25, 569−578

was azeotroped with toluene and dried under high vacuum for 30 min. Lyophilization yielded a white solid (185 mg) which was sufficiently pure by NMR. ^{1}H NMR (400 MHz, CD_3OD): δ 3.18 (t, J = 7.0 Hz, 2H), 2.92 (t, J = 7.8 Hz, 2H), 2.86 (t, J = 7.3 Hz, 2H), 2.30 (s, 3H), 2.17 (t, J = 7.3 Hz, 2H), 1.72 − 1.50 (m, 8H), 1.45 − 1.33 (m, 4H). ^{13}C NMR (100 MHz, CD_3OD): 197.7, 176.2, 40.7, 40.0, 37.0, 30.64, 30.61, 30.0, 29.8, 29.4, 28.3, 26.6, 24.8. ESI-QTOF MS m/z calculated for $C_{13}H_{26}N_2O_2S$ $[M+H]^+$ 275.1788, measured 275.1785.

Boc-C6-vc-PAB-MMAF (7). To a solution of maleimide-valine-citrullin-PAB-MMAF (8.8 mg, 6.61 μmol) in DMF (0.6 mL) was added 6.6 μL of a 0.1 M solution of triethylamine in DMF (0.66 μmol Et_3N), followed by the dropwise addition of a solution of *tert*-butyl (5-(6-mercaptohexanamido)pentyl)-carbamate **2** (3 mg, 9.02 μmol) in acetonitrile (0.3 mL). The reaction was stirred for 3 h while being monitored by HPLC (t_R = 18.5 min). After the reaction was complete it was diluted with water (2 mL) and purified with semipreparative RP HPLC with gradient system 1 (t_R = 10.3 min). The product was obtained as a white solid after lyophilization (8.7 mg, 79% yield). MS (ES+) m/z 832.44 $(M+2H)^{2+}$.

C6-vc-PAB-MMAF (8). Compound **7** (8 mg, 4.81 μm) was dissolved in an ice cold solution of dichloromethane/TFA 95:5 (8 mL). The reaction mixture was allowed to reach RT and stirred for 40 min (reaction monitoring by HPLC, t_R = 16.8 min) after which time the volatiles were removed under reduced pressure and dried under high vacuum. The residue was purified by semipreparative HPLC with gradient system 2, t_R = 11.7 min. The product was obtained as a white solid after lyophilization (4.86 mg, 65% yield). ESI-QTOF MS m/z calculated for $C_{79}H_{127}N_{13}O_{17}S$ $[M+2H]^{2+}$ 781.9670, measured 781.9667.

LC-ESI-MS Analysis. LC-ESI-MS analysis was performed on a Waters LCT Premier mass spectrometer. Prior to analysis, 10 μg of antibody was mixed with DTT (20 mM final concentration) and incubated at 37 °C for 30 min to separate heavy and light chains. Samples were chromatographed on an Aeris WIDEPORE XB-C18 column (3.6 μm, 100 mm × 2.1 mm; Phenomenex) heated to 80 °C using the following gradient: 0−1 min, 10−20% A; 1−30 min, 20−60% A; 30−31 min, 60−10% A (solvent A: 1:1 acetonitrile:isopropanol +0.1% formic acid, solvent B: water +0.1% formic acid) at a flow rate of 0.5 mL/min. The eluent was ionized using an electrospray source (ESI+). Data were collected with MassLynx 4.1 and deconvolution was performed using MaxEnt1. Unless stated otherwise, the following equations were used to estimate the relation between two mass peaks

$$\%\text{Mass Peak A} = \frac{\text{Intensity}_{\text{Mass Peak A}}}{\text{Intensity}_{\text{Mass Peak A}} + \text{Intensity}_{\text{Mass Peak B}}} \times 100$$

and

$$\%\text{Mass Peak B} = 100 - \%\text{Mass Peak A}$$

Deglycosylation of Antibodies. Antibody in phosphate buffered saline (PBS) was incubated with 6 U/mg protein of N-glycosidase F (PNGase F) from *Flavobacterium meningosepticum* (Roche, Switzerland) overnight at 37 °C. The enzyme was then removed by centrifugation-dialysis (Vivaspin MWCO 50 kDa, Vivascience, Winkel, Switzerland). The reaction was monitored by LC-ESI-MS.

Enzymatic Modification of Antibodies. One mg/mL deglycosylated antibody in PBS was incubated with 80 mol equiv (40 mol equiv per conjugation site) of the corresponding amine-functionalized chemical entity and 6 U/mL microbial transglutaminase (MTGase, Zedira, Darmstadt, Germany) overnight (16 h) at 37 °C. Excess substrate and the MTGase were removed by centrifugation-dialysis (for analytical purposes) or by size exclusion chromatography (SEC, Superdex 200 10/300 GL, GE Healthcare, 0.5 mL/min flow) for batch production.

Deacetylation of Protected Thiol Linkers and Maleimide−Thiol Conjugation. The method for deacetylation of the protected thiol linker was adapted from published procedures.[27] Briefly, deprotection stock solution containing 0.5 M hydroxylamine and 25 mM EDTA was prepared in PBS. The antibody−linker conjugate was combined with 10−40% v/v of the prepared deprotection stock solution in a final volume of 1 mL and the pH was adjusted to 7.7−8.8. The reaction mixture was then incubated for 2−6 h at RT or 37 °C (Table S1, Supporting Information) and subsequently purified on a desalting column. The protein was eluted with PBS containing 10 mM EDTA. Conditions for maleimide−thiol coupling were adopted from previously described experiments.[10] Briefly, the deglycosylated antibody was reacted with a 5-fold molar excess of maleimide-toxin (per thiol group) in PBS for 2 h at RT.

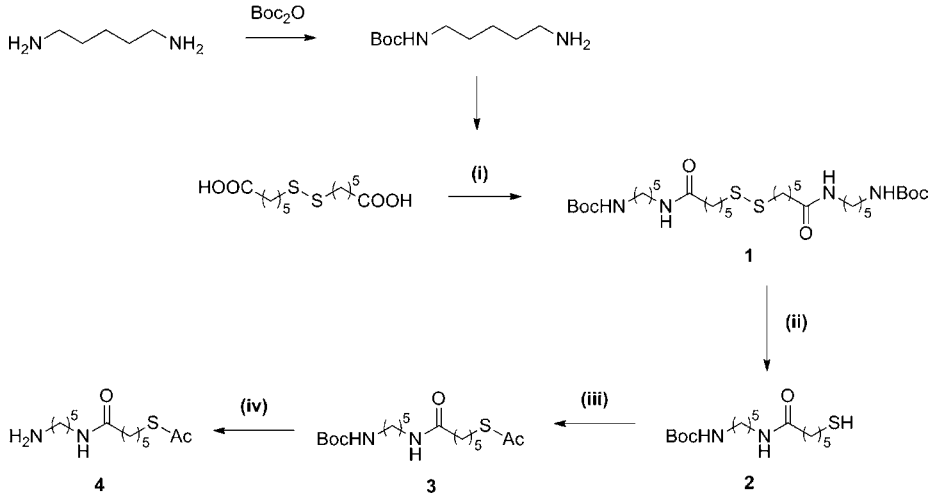
Strain-Promoted Azide−Alkyne Cycloaddition (SPAAC). 20 μM of azide-functionalized antibody was incubated with 50 to 200 μM (1.25 to 5 mol equiv per azide group) of DBCO-amine at RT for 0.5−6 h. Excess of DBCO-PEG4-vc-PAB-MMAE was removed by SEC. The final ADC was formulated in citrate buffer (20 mM sodium citrate, 1 mM citric acid, pH 6.6) containing 0.1% polysorbate 80 and 70 mg/mL trehalose.

Linker Cleavage Assay (Cathepsin B). Human liver cathepsin B (1.25 U/μL, Sigma Aldrich) was activated by incubating 3 μL of the protease with 84 μL water, 30 μL 0.5 M sodium acetate pH 5.0, and 6 μL 0.1 M dithiothreitol (DTT) at 37 °C. After 15 min, 28 μL ADC (1 mg/mL) was added and the reaction mixture was incubated at 37 °C for 6 h. Samples were taken after 2 and 6 h and analyzed by LC-ESI-MS.

Cell Lines and Antibodies. The human breast cancer cell lines BT-474 and SK-BR-3 were obtained from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 and McCoy's 5a (BioConcept), respectively. Media was supplemented with 10% fetal calf serum (FCS), 2 mmol/L glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone (BioConcept). The cells were maintained at 37 °C with 5% CO_2. Trastuzumab (Herceptin) was obtained from the University Hospital Zurich and a nonspecific chimeric IgG1 (Innate Pharma, France) was used as control IgG.

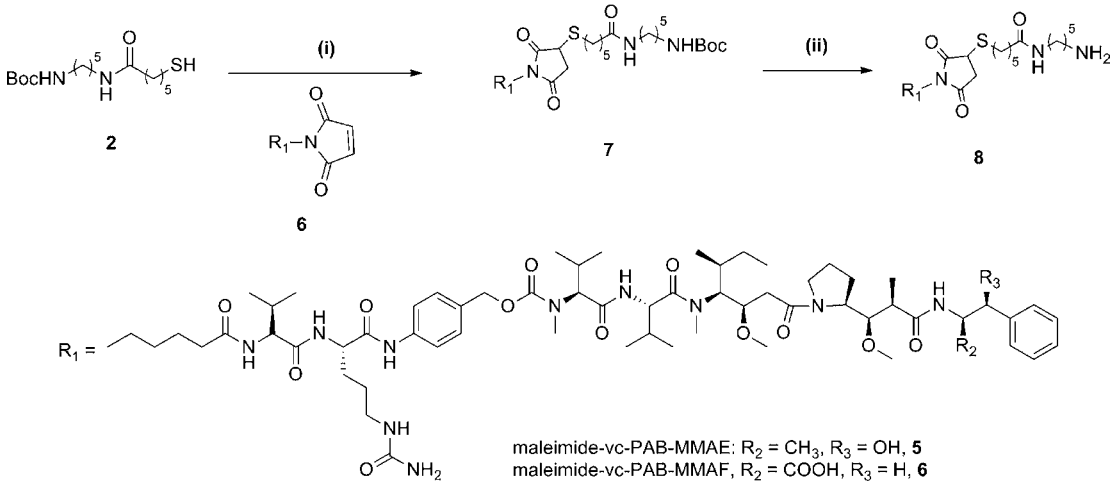
Fluorescence-Activated Cell Sorting (FACS). The binding ability of native antibodies and ADCs was determined by flow cytometry using an indirect fluorescent staining method. The cells were harvested by trypsin/EDTA, washed with PBS supplemented with 1% BSA (PBS/BSA) and incubated with 10 μg/mL antibody for 30 min at 4 °C. Cells were washed three times with PBS/BSA on ice and incubated with a goat anti-human IgG-FITC (1:200 dilution in PBS/BSA, Santa Cruz Biotechnology) for 30 min at 4 °C in the dark. The cells were washed again three times with PBS/BSA, resuspended in 200 μL PBS/BSA, and directly submitted to flow cytometry (Guava

Scheme 1. Synthesis of S-Protected Thiol-Linker C6-SAc Bearing a Primary Amine Function[a]



[a]Reagents: (i) HBTU, DIPEA, DMF, RT, 20 h, 93%; (ii) Bu_3P/H_2O, THF, RT, 1 h, 91%; (iii) Ac_2O/K_2CO_3, AcOEt, RT, 16 h, 90%; (iv) TFA, DCM, 0 °C to RT, 1 h, quantitative.

Scheme 2. Synthesis of C6-vc-PAB-MMAF and Structures of Maleimide-MMAE/F[a]



[a]Reagents: (i) DMF/MeCN 1:1, Et_3N, RT, 3 h, 79%; (ii) TFA, DCM, 0 °C to RT, 40 min, 65%.

easyCyte Flow Cytometer, Merck Millipore). Data was analyzed with FlowJo software (TreeStar Inc.).

Cell Toxicity Assay. Cells were plated in 96-well plates (~2000 and 3000 cells per well for SK-BR-3 and BT-474, respectively) and allowed to attach to the plates overnight at 37 °C. A serial dilution of wild-type antibody or ADC ranging from 66.6 nM (10 μg/mL) to 63.5 fM (9.5 pg/mL) was added and the cells were incubated at 37 °C. After 96 h, 30 μL of 5 mg/mL MTT-solution ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) was added to each well for 2−3 h at 37 °C in the dark. The supernatant was removed and the purple formazan crystals were dissolved in 200 μL DMSO. The absorption of the colored solution was measured at 506 nm on a Multilabel Plate Reader (VICTOR X3, Perkin-Elmer). The raw data was processed using GraphPad Prism 5.0 (GraphPad Software, San Diego, USA).

RESULTS AND DISCUSSION

Chemical Syntheses of the Spacer Entities. The synthesis of the C6-SAc linker (**4**) is shown in Scheme 1. Two equiv of N-Boc cadaverine were coupled to 6,6′-disulfanediyldihexanoic acid with HBTU in DMF to provide the disulfide **1** in excellent yield (93%). The starting material 6,6′-disulfanediyldihexanoic acid was synthesized from 6-bromohexanoic acid by following a reported procedure. [28] N-Boc cadaverine was synthesized from cadaverine according to a published protocol with slight modifications. [29] Treatment of compound **1** with Bu_3P/H_2O in THF afforded efficiently the free thiol **2** in 91% yield which was successively protected with an acetyl group by reacting it with acetic anhydride in AcOEt under basic conditions (**3**, 90% yield). Finally, cleavage of the Boc group in **3** with TFA in DCM afforded quantitatively linker **4** (C6-SAc) which was used for coupling with the antibody.

The synthesis of C6-vc-PAB-MMAF is depicted in Scheme 2. First, the Boc-protected-free thiol **2** was coupled with the MMAF toxin by reacting it with maleimide-vc-PAB-MMAF **6** in DMF to provide intermediate **7** in good yield (79%) after HPLC purification. Treatment of **7** with TFA in DCM and HPLC purification afforded C6-vc-PAB-MMAF in excellent purity (>98%).

dx.doi.org/10.1021/bc400574z | Bioconjugate Chem. 2014, 25, 569−578

Direct Enzymatic Conjugation of Linker-Toxins. We previously demonstrated that various small molecular weight cadaverin-derivatized substrates can be coupled by MTGase to a unique site on the heavy chains of deglycosylated mAbs. [21] Here, we tested if direct conjugation to chimeric and humanized IgG1 could also be achieved with cadaverin-derivatives of the auristatins MMAE and MMAF, both containing the cleavable dipeptide linker valine-citrulline. To this aim, we introduced two different spacers (C6 and PEG4) between the cleavable linker and the terminal amine and monitored the enzymatic conjugation by mass spectrometry.

The direct enzymatic conjugation reactions using a 40-fold excess C6-vc-PAB-MMAF (**8**) and PEG4-vc-PAB-MMAE (**9**) (Figure 3A) over conjugation site yielded approximately 50% and 80% of modified heavy chain for mAb-C6-MMAF and mAb-PEG4-MMAE, respectively. While PEG4-MMAE seems to be a better amine-donor for MTGase than C6-MMAF (Figure 4A), both toxin-derivatives are less reactive than the small molecular weight control substrate biotin-cadaverine. The coupling yield could theoretically be optimized by increasing the molar excess of the substrate or the units of MTGase. However, there are three critical issues that do not favor this direct enzymatic conjugation approach: (i) The acceptance of MTGase toward larger, amine-functionalized chemical entities such as toxins is unpredictable. Thus, reaction parameters have to be evaluated for each toxin individually. (ii) Lipophilic toxins might need to be dissolved in an organic solvent. However, these cosolvents can negatively influence the MTGase activity.[30] (iii) The large excess of toxin required for the reaction is another disadvantage with regard to large-scale production, since these cytotoxic agents are expensive.

Chemo-Enzymatic Method to Produce ADCs. To circumvent the problems outlined above, we developed a novel chemo-enzymatic approach whereby a small linker suitable for the enzymatic reaction by MTGase was used to introduce an additional thiol or azide functionality onto the antibody in a first step. The toxin, which was equipped with a functional group reactive toward the linker, could then be reacted with the antibody−linker conjugate to yield the desired ADC (Figure 2, bottom panel). We chose two different chemistries for this novel strategy: (i) Thiol-maleimide chemistry is a very popular chemistry to modify proteins via natural or engineered cyteines. Hence, numerous maleimide-functionalized chemical entities are commercially available, including toxins. (ii) Strain-promoted azide−alkyne cyclo-addition (SPAAC), a Cu(I)-free [2 + 3] cycloaddition reaction, is an emerging tool for protein modification.[31,32] Both chemistries should be fast and efficient and can be performed under mild conditions.

Thiol-Maleimide Approach (Figure 3B, top lane). First, we attached the commercially available thiol linker 6-amino-1-hexanethiol to the antibody by MTGase but observed major side products by LC-ESI-MS that could be assigned to dimerized linker attached to heavy chain (data not shown). Hence, we protected the thiol with an acetyl group to circumvent dimerization issues.

The protected thiol-linker C6-SAc (**4**) could be quantitatively conjugated to the antibody. We observed the expected mass shift for the heavy chain and there was no unmodified heavy chain remaining after the enzymatic reaction (Figure S1A, Supporting Information). In order to establish quantitative removal of the protection group, different conditions have been tested (Table S1, Supporting Information) with the

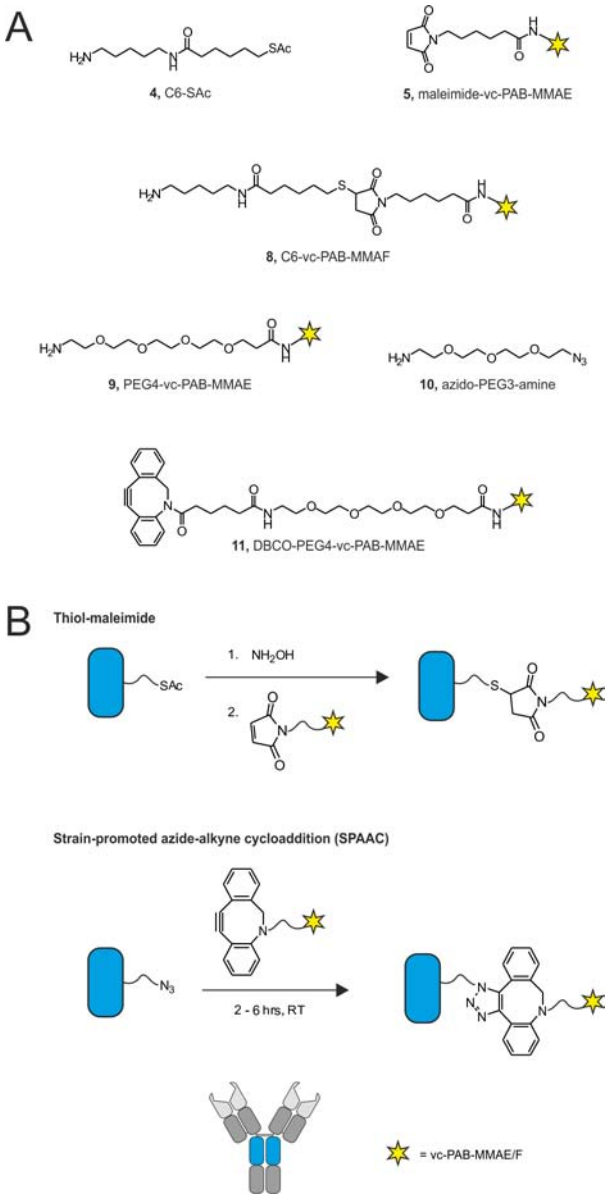


Figure 3. (A) Chemical structures of the different substrates for the enzymatic- and chemo-enzymatic approach. (B) General strategies for the chemo-enzymatic approaches. The thiol linker (**4**) and the azide linker (**10**) were enzymatically conjugated to the heavy chain (HC, cyan rectangle) of the antibody. Thiol-maleimide chemistry (top panel): The protected thiol was deacetylated under basic conditions. The free thiol was then further reacted with the maleimide-functionalized toxin (**5**) to yield the desired ADC. SPAAC reaction (bottom panel): The azide was reacted with the DBCO-functionalized toxin (**11**) in a Cu(I)-free [2 + 3] cycloaddition under mild conditions to yield the final ADC.

antibody−C6-SAc conjugate. However, residual acetylated heavy chain species were detected in all of the conditions tested. We reacted the antibody−C6-SH/antibody−C6-SAc conjugate mixture with a 3-fold molar excess of maleimide-vc-PAB-MMAE and found approximately 80−90% heavy chains with the desired mass shift (Figure S1B, Supporting Information). While these results demonstrate the potential of this approach as a tool for the production of homogeneous ADC, the deacetylation of the thiol prevents this method from being entirely successful. Additionally, two aspects have to be

dx.doi.org/10.1021/bc400574z | *Bioconjugate Chem.* 2014, 25, 569−578

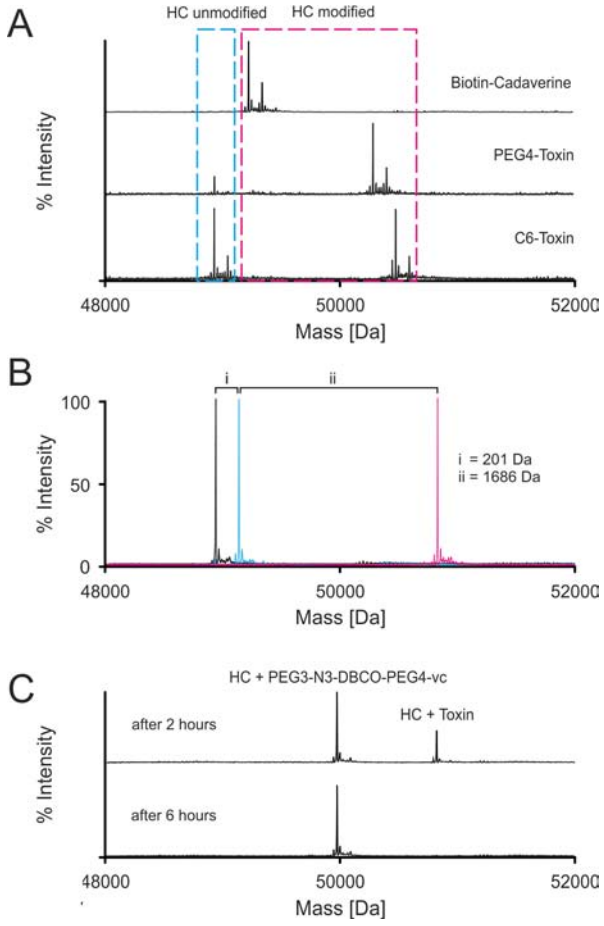


Figure 4. Deconvoluted mass spectra of different reactions. The smaller peaks with an increased mass correspond the TFA adduct of the main peak. (A) The PEG4-Toxin seems to be a better amine donor for MTGase than C6-Toxin because there is less unmodified heavy chain (HC, cyan dashed box) left after the enzymatic reaction. However, no unmodified HC (cyan dashed box) but only heavy chain with the expected mass shift of 311 Da (328 Da [molecular weight of biotin-cadaverine] − 17 Da [ammonia] = 311 Da; magenta dashed box) could be found after the enzymatic reaction with biotin-cadaverine. This indicates that the linker length and the size of the entire chemical entity have a influence on the enzymatic reaction outcome. (B) The SPAAC reaction was monitored by LC-MS and showed expected mass shifts between (i) unmodified HC (black) and HC after the enzymatic reaction with MTGase and amine-PEG3-azide linker (cyan) of 201 Da (218 Da [molecular weight of amine-PEG3-azide linker] − 17 Da [ammonia] = 201 Da) and (ii) HC + azide-linker (cyan) and the HC after SPAAC reaction with DBCO-PEG4-vc-PAB-MMAE (magenta) of 1686 Da ([molecular weight of DBCO-PEG4-vc-PAB-MMAE]). Moreover, yields of both the enzymatic (1st step) and the chemical (second step) reaction are quantitative (>95%). (C) The toxin was completely cleaved after 6 h by cathepsin B and a single peak that corresponds to HC + PEG3-N3-DBCO-PEG4-vc could be observed.

considered when maleimide−thiol chemistry is used (i) immunoglobulin aggregation may be influenced by free thiols that can form disulfide bonds between antibodies, and (ii) depending on the site of conjugation, maleimide exchange processes can influence the performance of the antibody conjugate.[11]

Strain-Promoted Azide−Alkyne Cycloaddition (SPAAC, Figure 3B, bottom lane). The enzymatic reaction with the small linker azido-PEG3-amine (**10**) quantitatively yielded the desired antibody conjugate (Figure 4B). DBCO-amine was used as a model substrate to determine optimal reaction conditions for the Cu(I)-free [2 + 3] cycloaddition. We varied the molar equiv of DBCO-amine and monitored the progress by analyzing reaction aliquots after different time intervals by LC-ESI-MS. The SPAAC reaction was very fast and yielded homogeneous antibody conjugates after 6 h at RT with a minimal molar excess of 1.25 equiv per azide group (Figure S2, Supporting Information). We applied these conditions to attach DBCO-PEG4-vc-PAB-MMAE (**11**) and obtained uniform ADCs (Figure 4B).

These results impressively demonstrate the ability of our chemo-enzymatic approach to produce homogeneous ADCs with respect to site-specificity and stoichiometry. A defined DAR is important for ADCs since a heterogeneous drug load can have a negative influence on the *in vivo* properties.[8,9] Moreover, ADCs obtained by SPAAC conjugation are potentially more stable *in vivo* than thiol-maleimide coupled ADCs, because of maleimide exchange processes with reactive thiols on serum proteins.[11] Furthermore, the minimal excess of expensive toxin used is clearly advantageous with respect to scaled-up production of ADCs. Our method can also be applied to a broad variety of IgGs since the site of modification is located within the constant heavy chain domain and is conserved in human IgG1−4, murine IgG1/3, and rat IgG1/2a−c.

Deglycosylation of the antibody is, however, inevitable when exploiting our chemo-enzymatic approach since the site of modification (Q295) is adjacent to the glycosylation site (N297). It has been reported that carbohydrate removal influences the structural stability of antibodies thereby leading to an increased aggregation rate.[33] Nevertheless, several aglycosylated antibodies are currently evaluated in clinical trials.[34] Moreover, the lack of the carbohydrate moiety can even be advantageous since hepatic toxicities of ADCs seem to be driven by interactions of the antibody glycans with the mannose receptor, resulting in off-target cellular uptake.[35] In addition, abrogated FcR+ interaction can potentially be advantageous as a lower uptake by FcR+ cells can lead to a limited off-target toxicity and improve tumor specific targeting.[36,37]

Biological Evaluation of the ADC. In order to prove the applicability of our novel technology we selected two different antibodies, a humanized anti-HER2/neu IgG1 (Trastuzumab, Herceptin) and a nonspecific IgG1 (control IgG), and transformed them into ADCs according to the established protocol. These conjugates were then used to assess the biological activity.

Mass spectrometric analysis of trastuzumab-PEG3-N3-DBCO-PEG4-vc-PAB-MMAE (trastuzumab-MMAE) after the incubation with human liver cathepsin B revealed complete cleavage of the toxin after 6 h (Figure 4C). These results demonstrate that the introduction of a PEG4 spacer or a DBCO functionality does not inhibit the enzymatic cleavage of the dipeptide valine-citrulline.

Next, we examined the ability of trastuzumab-MMAE to bind to HER2/neu by flow cytometry on HER2/neu+ cell lines (BT-474, SK-BR-3) and observed binding of the ADC to both cell lines (Figure 5A, left). This result is not surprising because the site of drug attachment (CH_2 domain) is distant from the antigen-binding site. In addition, no variant of the control IgG1 (unmodified and ADC) bound to BT-474 or SK-BR-3 cells (Figure 5A, right). Finally, the ADC was tested for cytotoxicity

dx.doi.org/10.1021/bc400574z | *Bioconjugate Chem.* 2014, 25, 569−578

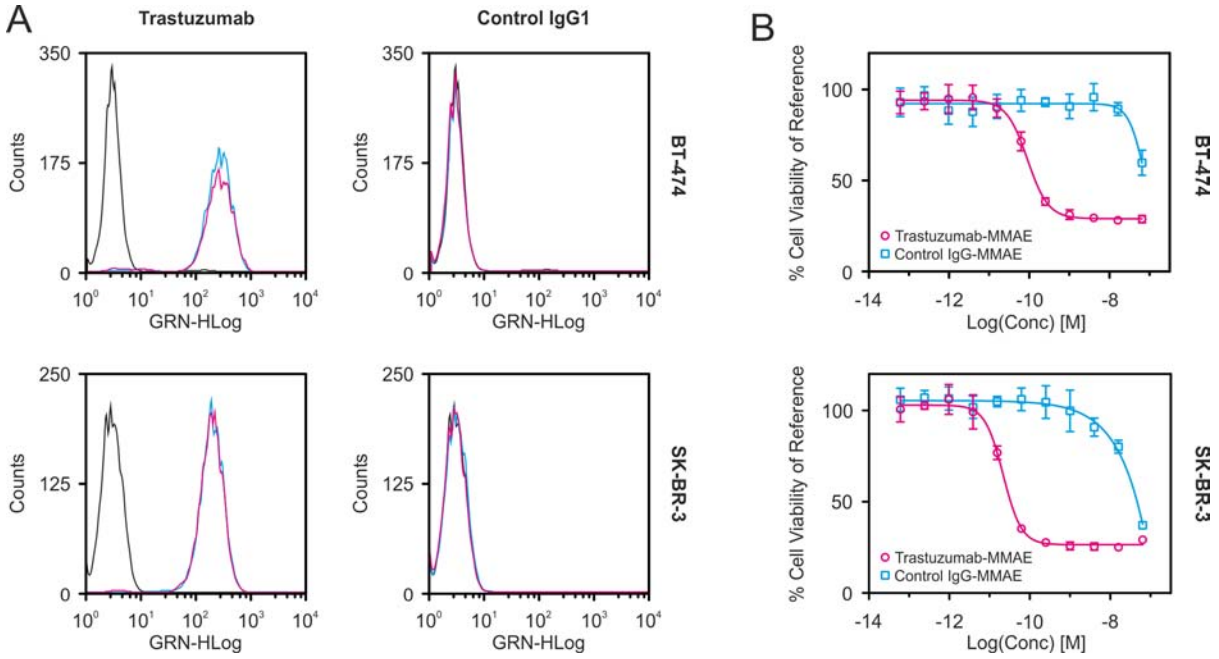


Figure 5. (A) Fluorescence-activated cell sorting (FACS) analysis of trastuzumab/control IgG1 wild-type (cyan) and trastuzumab-MMAE/control IgG1-MMAE (magenta) on human breast cancer cell lines BT-474 (top) and SK-BR-3 (bottom). (B) Trastuzumab-MMAE (circles) effectively inhibits cell proliferation of both BT-474 and SK-BR-3 cells, whereas the control IgG1-MMAE (square) shows cytotoxic effect only at high concentrations.

and selectivity to prove its functionality. Trastuzumab-MMAE effectively killed BT-474 and SK-BR-3 cells with an IC_{50} of 89.0 pM and 21.7 pM, respectively, whereas the control IgG-PEG3-N3-DBCO-PEG4-vc-PAB-MMAE (control IgG-MMAE) did not kill BT-474 or SK-BR-3 cells (IC_{50} > 700-fold higher). These IC_{50} values are comparable with published values where a similar cell toxicity assay setup was used.[12] However, a nonspecific effect on cell proliferation was observed with the highest ADC concentration (10 μg/mL, Figure 5B). As an inhibitory effect arising from the antibody itself cannot be ignored, the impact of both unconjugated trastuzumab and control antibody on cell proliferation was evaluated in parallel on both cell lines. While the control IgG1 had no effect on cell proliferation, trastuzumab influenced the proliferation of both BT-474 and SK-BR-3 cells (Figure S3, Supporting Information). Nevertheless, trastuzumab-MMAE showed an approximately 10-fold lower IC_{50} for both cell lines, clearly demonstrating the enhanced potency of the ADC in comparison to the unmodified antibody.

CONCLUSION

In this article, we have described different approaches to endow antibodies with MMAE in a site-specific and stoichiometric uniform manner. The chemo-enzymatic approach using SPAAC chemistry was superior to the other evaluated methods with respect to product homogeneity and amount of MMAE required. The fact that a minimal amount of toxin is needed using a two-step, chemo-enzymatic approach can be a crucial argument with regard to large-scale production of ADCs because of reduced costs and prevention of hazardous waste. Our results demonstrate the versatility and potential of this conjugation method. It not only allows the transformation of virtually any IgG1 into a functional ADC, but also enables a direct comparison of, e.g., different antibodies, linker systems, or toxins, because of the known site of drug attachment and the uniform stoichiometry. Therefore, the chemo-enzymatic procedure is a powerful tool for both ADC production and development of next generation ADCs.

ASSOCIATED CONTENT

Supporting Information

LC-ESI-MS spectra of thiol-maleimide approach, reaction conditions for the deacetylation reaction of protected C6-SAc (**4**), reaction kinetics of SPAAC and cell toxicity assays with nonarmed trastuzumab on SK-BR-3 and BT-474 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: roger.schibli@psi.ch.

Notes

The authors declare the following competing financial interest(s): Parts of the project were financially supported by Innate Pharma. Schibli, Dennler, Fischer, Chiotellis do not have shares with Innate Pharma. Bregon, Lhospice, Gauthier, Belmant, Romagne do have shares with Innate Pharma.

ACKNOWLEDGMENTS

The authors thank Alain Blanc and Susan Cohrs for technical assistance with the LC-ESI-MS and cell culture, respectively. We also thank Laura Bailey for reviewing the manuscript. This work was funded by the Swiss National Science Foundation (Grant No. 132611).

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