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Enzymatic Prenylation and Oxime Ligation for the Synthesis of Stable and Homogeneous Protein–Drug Conjugates for Targeted Therapy

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Abstract: *Targeted therapy based on protein–drug conjugates has attracted significant attention owing to its high efficacy and low side effects. However, efficient and stable drug conjugation to a protein binder remains a challenge. Herein, a chemoenzymatic method to generate highly stable and homogenous drug conjugates with high efficiency is presented. The approach comprises the insertion of the CaaX sequence at the C-terminal end of the protein binder, prenylation using farnesyltransferase, and drug conjugation through an oxime ligation reaction. MMAF and an EGFR-specific repebody are used as the antitumor agent and protein binder, respectively. The method enables the precisely controlled synthesis of repebody–drug conjugates with high yield and homogeneity. The utility of this approach is illustrated by the notable stability of the repebody–drug conjugates in human plasma, negligible off-target effects, and a remarkable antitumor activity in vivo. The present method can be widely used for generating highly homogeneous and stable PDCs for targeted therapy.*

Over the past few decades, considerable advances have been made in the development of therapeutic agents for various diseases, including cancers, and a number of therapeutic agents, including small-molecule drugs and monoclonal antibodies, have been clinically used.[1] Despite the widespread use of monoclonal antibodies and chemical drugs, their efficacy and selectivity remain a challenge.[2] In recent years, a new type of targeted therapy that is based on the conjugation of cytotoxic drugs to protein binders has attracted significant attention.[3] Drug-conjugated protein binders are expected to effectively deliver cytotoxic agents into specific cancer cells through receptor-mediated internalization followed by drug release, leading to a significantly improved therapeutic window owing to the increased selectivity and potency of the chemical drugs. Two antibody–drug conjugates (ADCs) are being clinically used, and a number of ADCs are currently undergoing clinical trials.[3a] Antibodies generally show low tissue penetration owing to their large size, and small non-antibody scaffolds have recently been used for conjugating drugs to enhance the delivery efficiency.[4]

The homogeneity and stability of protein–drug conjugates (PDCs) have a profound effect on their pharmacological properties, such as the plasma half-life, therapeutic efficacy, and off-target toxicity.[5] However, coupling reactions of amines with NHS-ester or thiols with maleimide, which have been widely employed for the synthesis of drug conjugates, have mostly resulted in heterogeneous products with different stoichiometries, leading to unwanted toxicity and increased clearance. Furthermore, thiol–maleimide linkages are prone to hydrolysis and undergo a reversible exchange reaction with serum protein thiols or free cysteine through a retro-Michael reaction, causing premature drug release and inefficient drug delivery to the target site.[6] To overcome these drawbacks, site-specific and stoichiometric conjugation methods have been attempted using substituted cysteine residues or unnatural amino acids, along with enzymes such as transglutaminase or sortase.[7] Despite many advances, however, an efficient and versatile method to generate stable and homogeneous PDCs for highly efficacious targeted therapy has yet to be developed.

Herein, we present a chemoenzymatic method to generate highly homogeneous and stable protein–drug conjugates in a site-specific manner. Our approach comprises the insertion of the CaaX sequence at the C-terminal end of the protein binder, prenylation using farnesyltransferase (FTase), and selective drug conjugation through an oxime ligation reaction (Scheme 1). We previously developed a non-antibody scaffold, called a "repebody", which is composed of leucine-rich repeat (LRR) modules.[8] In this study, a repebody with high affinity for an epidermal growth factor receptor (EGFR) was used as the protein binder. Monomethyl auristatin F (MMAF) was employed as the cytotoxic drug.[9] Our method enables the simple and efficient conjugation of MMAF to the protein binder, yielding homogeneous and stable repebody–drug conjugates (RDCs). We investigated

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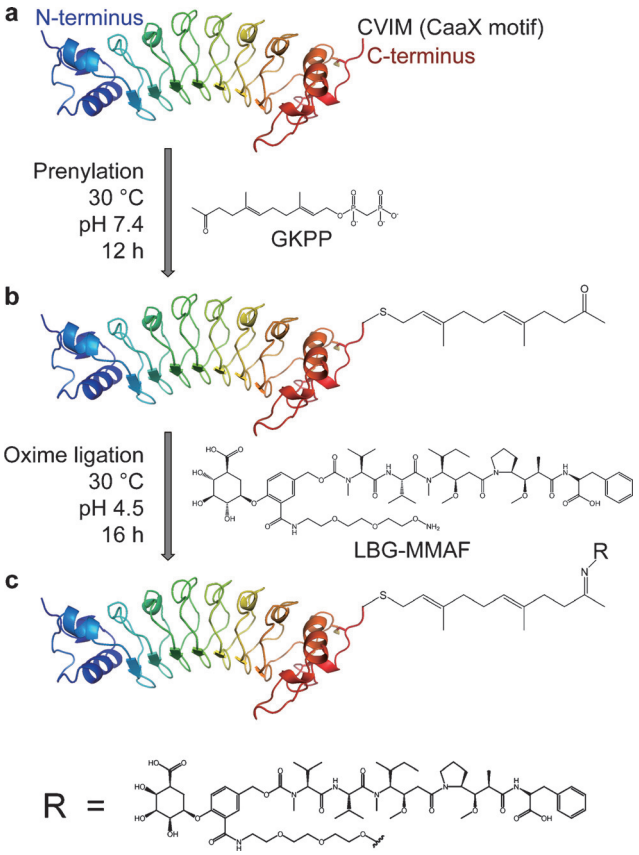

Scheme 1. Chemoenzymatic synthesis of RDCs. a) Overall structure of a repebody with a CaaX motif (Cys-Val-Ile-Met) and prenylation of the CaaX repebodies with geranyl ketone pyrophosphate (GKPP). b) Site-specific conjugation of β-glucuronide-linked and aminooxylated MMAF (LBG–MMAF) to prenylated repebodies through oxime formation. c) Schematic representation of the repebody–MMAF conjugates.

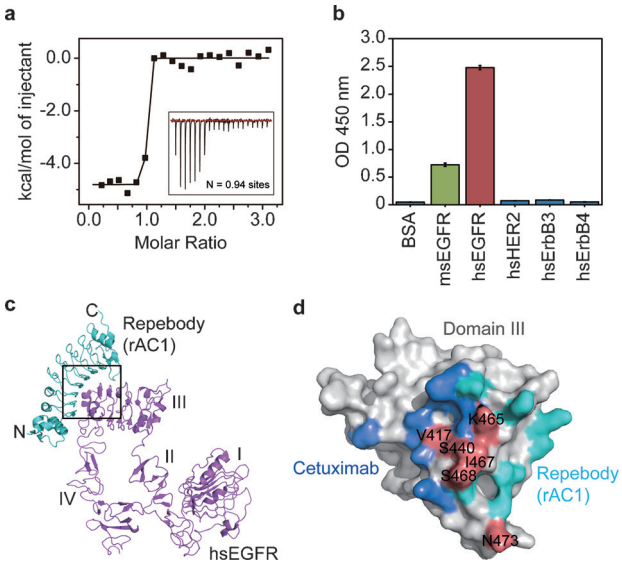

Figure 1. Characterization of the EGFR-specific repebody. a) Calorimetric data for the titration of repebody rEgH9 into hsEGFR solution. b) Specificity of the repebody towards EGFR as determined by phage ELISA analysis. Bovine serum albumin (BSA), mouse soluble EGFR ectodomain (msEGFR), human soluble EGFR2 ectodomain (hsHER2), human soluble EGFR3 ectodomain (hsErbB3), and human soluble EGFR4 ectodomain (hsErbB4) were coated onto a 96 well plate at a concentration of 10 μg mL^{-1}. Error bars indicate the standard deviations in triplicate experiments. c) Structure of tethered hsEGFR in complex with a repebody (PDB No. 4UIP). Each domain of the EGFR is indicated by Roman numerals. d) Repebody (rAC1) epitope compared with the cetuximab epitope on the EGFR domain III. The overlapping region is highlighted in red.

the cytotoxic and anti-tumor activities of the RDCs both in vitro and in vivo.

We previously constructed a synthetic repebody library for a phage display.[8a] Using this library, we selected repebodies that are specific for the human soluble EGFR ectodomain (hsEGFR), which is known to be overexpressed in numerous cancers.[10] Among them, a repebody (rA11) with an apparent binding affinity of 92 nM for hsEGFR was chosen, and its binding affinity was increased using a modular evolution approach (Supporting Information, Figure S1 and Table S1).[8b] The resulting repebody (rEgH9) was shown to have a sub-nanomolar affinity ($K_D = 301$ pM) and high specificity for hsEGFR with negligible cross-reactivity against other EGFR family members (Figure 1 a, b). Deletion of the EGFR domains I and II is the most common type of mutation in cancer patients, resulting in an acquired drug resistance.[10] To obtain insight into the binding mode of the repebody, we conducted a competitive immunoassay using a monoclonal antibody, cetuximab, that targets the EGFR domain III.[11] The signal intensity significantly decreased in the presence of cetuximab (Figure S2), implying that the binding epitope of the repebody is located within the EGFR domain III. We assessed the targeting ability of the repebodies using various human cancer cells that expressed different levels of EGFR. Fluorescein-labeled repebodies exhibited distinct fluorescence signals for cancer cells with high EGFR expression levels (Figure S3), showing a close correlation with their binding affinities for EGFR (Figure S4). It is noteworthy that the repebody rEgH9 has a higher penetration ability than the monoclonal antibody cetuximab in vivo (Figure S5). This result implies that a repebody with a small size (ca. 30 kDa) can facilitate effective penetration and accumulation of a payload in tumor tissues.

To directly demonstrate the binding mode of a repebody to EGFR, we crystallized and determined the structure of the complex of repebody rAC1 and hsEGFR at 2.95 Å resolution (Figure 1 c). The repebody/hsEGFR complex is likely to be majorly sustained by hydrogen bonds and salt-bridge interactions (Figure S6a). Interestingly, hsEGFR was determined to be present in tethered form, and there was no significant conformational change of hsEGFR upon binding to the large surface of the repebody, and several residues overlapped between the EGF and repebody binding sites (Figure S6 b). It is therefore likely that the repebody inhibits the EGF/EGFR signaling pathway by blocking the binding of EGF to EGFR in a competitive manner and preventing a conformational change in EGFR (Figure S6 c, d). In contrast, the repebody is shown to overlap with cetuximab at a number of epitope residues in the EGFR domain III (Figure 1 d), which explains the immunoassay result shown in Figure S2.

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As the first step towards drug conjugation, we incorporated a functionalized lipid moiety into the repebody rEgH9 using FTase, which specifically recognizes consensus CaaX motifs and efficiently catalyzes prenylation through the covalent attachment of isoprenoid analogues.[12] Therefore, we constructed a CaaX–repebody through the addition of a flexible glycine linker and a CaaX sequence of Cys-Val-Ile-Met (CVIM) at the C-terminal end of the repebody. The CaaX–repebody was revealed to be stably overexpressed in a soluble monomeric form without aggregation and inactivation. We synthesized a geranyl ketone pyrophosphate (GKPP) to site-specifically introduce a bioorthogonally reactive group into the CaaX–repebody for chemoselective drug conjugation, and conducted an orthogonal functionalization of the repebody through prenylation (Scheme 1 a). Following the enzymatic reaction, the resulting repebodies were subjected to hydrophobic-interaction chromatography/high-performance liquid chromatography (HIC-HPLC) to evaluate the prenylation efficiency and product homogeneity. As shown in Figure 2 a, the prenylated repebody was eluted as a single homogenous peak corresponding to approximately 98% of the total peak area, showing a significant shift in the retention time ($R_t = 14.0$ min) compared with the CaaX–repebody ($R_t = 10.2$ min). This result indicates a conjugation efficiency of greater than 95% and a negligible amount of a heterogeneous mixture. Owing to the free cysteine residue of the CVIM sequence, unmodified CaaX–repebodies will be prone to dimerization through interchain disulfide bonds under oxidative conditions. Nonetheless, the lipidated repebodies were confirmed to have no reactive cysteine residues through a non-reducing SDS-PAGE analysis (Figure 2 b). It is likely that FTase-catalyzed prenylation is effective for the incorporation of a unique functional group into the protein binder.

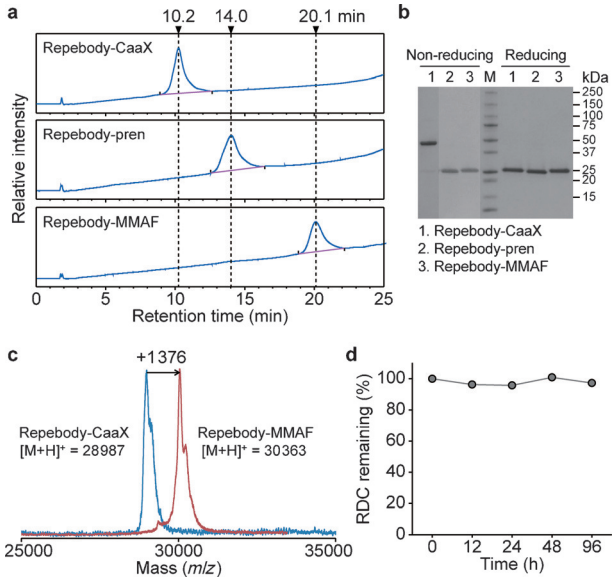

Figure 2. Homogeneity of synthesized RDCs. a) HIC-HPLC profiles of the naïve CaaX–repebodies (repebody–CaaX), prenylated repebodies (repebody–pren), and repebody–MMAF conjugates (repebody–MMAF). The profiles were normalized through signal intensity standardization. b) SDS-PAGE analysis of RDCs under non-reducing and reducing conditions. Protein bands of 25 kDa and 50 kDa indicate the monomer and dimer forms of the repebodies, respectively (left; non-reducing conditions). c) MALDI-TOF analysis of the CaaX repebodies (blue) and the repebody–MMAF conjugates (red). The peak corresponding to the CaaX–repebodies shifted by approximately 1376 upon drug conjugation, which corresponds to the attachment of only one molecule of LBG–MMAF per prenylated repebody. d) In vitro plasma stability of the RDCs. The percentage of remaining RDCs was calculated based on a comparison of the relative values of MMAF (serum) and repebody (serum), as shown in Figure S8 b.

An oxime bond (C=N–O) is known to be highly resistant to hydrolytic cleavage in aqueous solvent at physiological pH, and oxime formation is a suitable orthogonal reaction for efficiently synthesizing chemically stable drug conjugates.[13] We synthesized β-glucuronide-linked and aminooxylated MMAF (LBG–MMAF) for the production of RDCs through a chemoselective oxime reaction. MMAF, a microtubule-disrupting agent, has been widely exploited as a promising payload owing to its extremely high potency.[9] The glucuronide linkage is known to be stable in the blood and to be specifically cleaved by lysosomal β-glucuronidase.[14] Then, we conducted an oxime ligation between the ketone moieties of the prenylated repebodies and the hydroxylamines of LBG–MMAF (Scheme 1 b). The resulting repebody–MMAF conjugates were purified and characterized through HIC-HPLC and SDS-PAGE analysis. The repebody–MMAF conjugates were eluted as a single peak (97.4% of the total area) at a retention time of 20.1 min, and detected in their monomeric form (Figure 2 a, b). We checked the drug-loading ratio of the RDCs by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Figure S7). Figure 2 c shows the representative mass spectra of intact CaaX–repebody and repebody–MMAF conjugates. The peak at m/z 28987 ($[M+H]^+$), which corresponds to CaaX–repebody ($M_w = 28977$), was shifted to m/z 30363 after MMAF conjugation. The shifted peak is in good agreement with the calculated mass of the repebody–MMAF conjugates ($M_w = 30399$; drug/repebody ≈ 1.0). Based on these results, our approach seems to be applicable to the simple and efficient synthesis of RDCs with high homogeneity.

Owing to the high cytotoxicity of drugs, the premature release of free drug from the conjugates can result in severe systemic toxicity and a narrow therapeutic index. Therefore, the linker stability during circulation has been considered to be the most critical issue in the development of drug conjugates with high safety and efficacy.[3] We evaluated the plasma stability of the RDCs by monitoring the remaining repebody–MMAF conjugates after incubation with human serum for different periods of time (Figure S8). The repebody–MMAF conjugates were shown to have a high stability without any obvious changes when kept in the buffer. Next, the stability of the RDCs in serum was assessed by comparing the results of two independent ELISA experiments using MMAF and a repebody as the target molecules, respectively. The repebody–MMAF conjugates showed remarkable stability in human serum in vitro, with a serum half-life exceeding 96 hours (Figure 2 d). Our results demonstrate that the chemoenzymatic method enables the synthesis of highly stable RDCs in a site-specific manner.

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To evaluate the effect of the binding affinity on the cytotoxic activity of RDCs, we synthesized three different drug conjugates using repebodies with different binding affinities for EGFR, and examined their cytotoxicity towards EGFR-overexpressing HCC827 cells (Figure S9 and Table S1). The rEgH9–MMAF conjugates exhibited the highest cytotoxicity compared with the other two low-affinity conjugates. This result indicates that the binding affinity of a repebody for EGFR is critical to the cytotoxic activity of the RDCs, and optimizing the binding affinity can enhance the cytotoxic activity of drug conjugates. To investigate the relationship between the cell-surface expression level of EGFR and the cytotoxicity of the RDCs, we incubated three cancer cell lines expressing different levels of EGFR (Figure 3a) with various concentrations of the rEgH9–MMAF conjugates for three days (Figure 3b–d). The repebody–MMAF conjugates showed strong cytotoxic effects towards A431 and HCC827 cells in a dose-dependent manner, resulting in effective half-maximal concentrations (EC_{50}) of 1.4 nM and 0.072 nM, respectively (Table 1). This result indicates that the repebody–MMAF conjugates have a much higher cytotoxicity than free, cell-impermeable MMAF ($EC_{50} = 117.9$ nM) and naked repebodies (rEgH9: $EC_{50} = 17.2$ nM) towards HCC827 cells. Interestingly, the repebody–MMAF conjugates showed much higher potency in HCC827 cells than in A431 cells. This result seems to be due to the fact that HCC827 cells express constitutively internalized oncogenic EGFRs and consequently have an increased uptake of repebody–MMAF conjugates.[15] The cytotoxicity of the repebody–MMAF conjugates towards MCF7 cells was shown to be negligible even at a high dose (> 200 nM; Table 1). Our results demonstrate that RDCs can efficiently deliver a potent anticancer drug to the target cells in a receptor-specific manner, minimizing off-target effects.

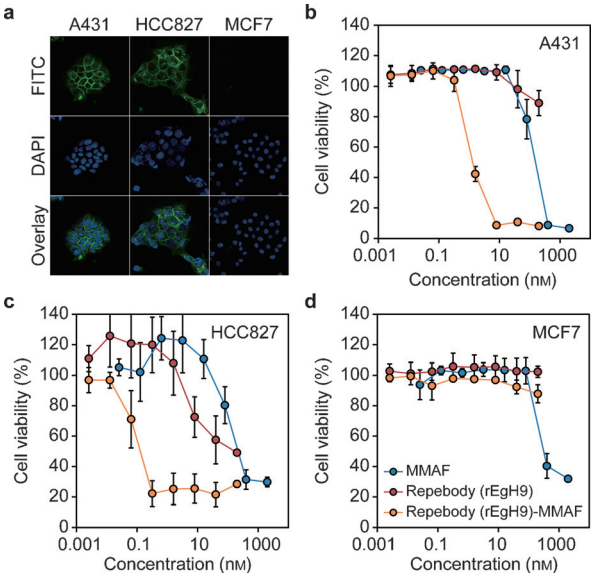

Figure 3. In vitro selective cytotoxicity of the RDCs. a) Confocal fluorescence images with fluorescein-labelled repebody (rEgH9). Cells were treated with 1 μg mL^{-1} of the labelled repebody for three hours, and subjected to confocal analysis. A431 (high EGFR expression, left), HCC827 (high EGFR expression, middle), and MCF7 (low EGFR expression, right) cells were used. The viabilities of the b) A431, c) HCC827, and d) MCF7 cells were determined after treatment with MMAF alone, naked repebodies, or the rEgH9–MMAF conjugates at different concentrations.

Table 1: EC_{50} values of MMAF, naked repebodies, and the repebody–MMAF conjugates in three different cancer cells.[a]

	EC_{50} [nM]		
	A431	HCC827	MCF7
MMAF	163.5 ± 44.6	117.9 ± 57.1	256.5 ± 19.3
rEgH9	–	17.2 ± 5.5	–
rEgH9–MMAF	1.4 ± 0.1	0.072 ± 0.01	–

[a] The EC_{50} values were estimated from Figure 3 and represent the concentration at which the cell viability was reduced by 50% in the presence of MMAF, naked repebodies (rEgH9), or rEgH9–MMAF conjugates.

We also evaluated the antitumor activity of the rEgH9–MMAF conjugates in xenograft mice using HCC827 cells. When the tumor volume reached 110 to 130 mm^3, the mice were subjected to daily intravenous injections (10 mg kg^{-1}) of the repebody–MMAF conjugates or naked repebody (rEgH9) for six days (Figure 4a). As a positive control, cetuximab was used. The repebody–MMAF conjugates showed a significant tumor regression response (33.4% residual tumor on day 20, $^{***}P < 0.001$) compared with the naked repebody (83.4% residual tumor on day 20, $P > 0.05$) and cetuximab. No significant adverse effects were detected in the treated mice on day 20 ($P > 0.05$), with the exception of transient weight loss (Figure 4b).

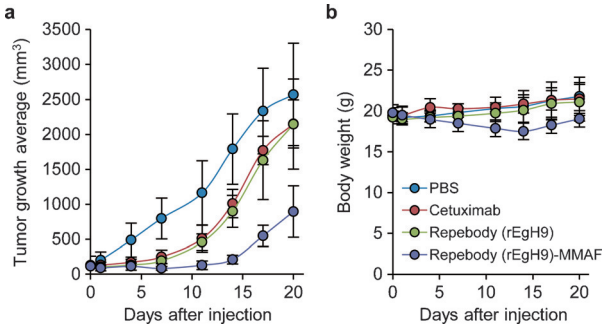

Figure 4. In vivo antitumor activity of the RDCs. a) Nude xenograft mice (HCC827) were administered with the rEgH9–MMAF conjugates, naked repebodies, or cetuximab (10 mg kg^{-1}) intravenously every day for six days after tumor establishment. The tumor size was measured every third day for 20 days (mean ± SD; $n = 6$). b) Changes in the mouse body weight. After administration, the body weights of each mouse were measured every third day.

In summary, we have developed a simple and efficient chemoenzymatic method that makes use of a FTase-catalyzed process and an oxime ligation reaction for generating highly stable and homogeneous repebody–drug conjugates in a site-specific manner. Repebody–MMAF conjugates were shown to have negligible off-target effects in vitro and a remarkable

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antitumor activity in xenograft mice, which implies that RDCs can be developed into safe and efficacious anticancer agents for targeted therapy. However, the repebody–drug conjugates exhibited imperfect suppression of the tumor growth. Considering the short half-life of a repebody in the blood, an optimization of the treatment conditions and an extension of the half-life of the repebodies will be necessary to further improve the therapeutic efficacy of the RDCs. The efficacy of the targeted therapy is largely dependent on the intracellular delivery efficiency of the cytotoxic payloads by a protein binder. In particular, cell penetration and the internalization of protein binders have been considered as key factors that affect the therapeutic efficacy of a drug. Considering the targeting ability, it is likely that a repebody can be effectively used as a protein binder for developing drug conjugates. In contrast to conventional conjugation methods, which usually result in heterogeneous mixtures of drug conjugates with various drug-to-protein ratios, our approach enables the controlled and efficient conjugation of a cytotoxic drug to a protein binder in a stoichiometric manner. Owing to its distinct advantages, our method can be generally used for drug conjugation to a wide range of protein binders including antibodies, facilitating the development of targeted therapies with high efficacies and low off-target effects.

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[1] a) M. X. Sliwkowski, I. Mellman, *Science* **2013**, *341*, 1192–1198; b) S. Nussbaumer, P. Bonnabry, J.-L. Veuthey, S. Fleury-Souverain, *Talanta* **2011**, *85*, 2265–2289.
[2] C. Holohan, S. Van Schaeybroeck, D. B. Longley, P. G. Johnston, *Nat. Rev. Cancer* **2013**, *13*, 714–726.
[3] a) R. V. J. Chari, M. L. Miller, W. C. Widdison, *Angew. Chem. Int. Ed.* **2014**, *53*, 3796–3827; *Angew. Chem.* **2014**, *126*, 3872–3904; b) C. Hess, D. Venetz, D. Neri, *MedChemComm* **2014**, *5*, 408–431.
[4] a) T. Yokota, D. E. Milenic, M. Whitlow, J. Schlom, *Cancer Res.* **1992**, *52*, 3402–3408; b) M. A. Firer, G. Gellerman, *J. Hematol. Oncol.* **2012**, *5*, 70; c) V. Cortez-Retamozo, N. Backmann, P. D. Senter, U. Wernery, P. D. Baetselier, S. Muyldermans, H. Revets, *Cancer Res.* **2004**, *64*, 2853–2857; d) M. Simon, R. Frey, U. Zangemeister-Wittke, A. Plückthun, *Bioconjugate Chem.* **2013**, *24*, 1955–1966.
[5] S. Panowski, S. Bhakta, H. Raab, P. Polakis, J. R. Junutula, *mAbs* **2014**, *6*, 34–45.
[6] R. P. Lyon et al., *Nat. Biotechnol.* **2014**, *32*, 1059–1062.
[7] a) B. M. Paterson, K. Alt, C. M. Jeffery, R. I. Price, S. Jagdale, S. Rigby, C. C. Williams, K. Peter, C. E. Hagemeyer, P. S. Donnelly, *Angew. Chem. Int. Ed.* **2014**, *53*, 6115–6119; *Angew. Chem.* **2014**, *126*, 6229–6233; b) J. Y. Axup et al., *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 16101–16106; c) S. Jeger, K. Zimmermann, A. Blanc, J. Grünberg, M. Honer, P. Hunziker, H. Struthers, R. Schibli, *Angew. Chem. Int. Ed.* **2010**, *49*, 9995–9997; *Angew. Chem.* **2010**, *122*, 10191–10194; d) J. R. Junutula et al., *Nat. Biotechnol.* **2008**, *26*, 925–932.
[8] a) S.-C. Lee et al., *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 3299–3304; b) J. Lee et al., *Mol. Ther.* **2014**, *22*, 1254–1265.
[9] S. O. Doronina et al., *Bioconjugate Chem.* **2006**, *17*, 114–124.
[10] P. Seshacharyulu, M. P. Ponnusamy, D. Haridas, M. Jain, A. K. Ganti, S. K. Batra, *Expert Opin. Ther. Targets* **2012**, *16*, 15–31.
[11] S. Li, K. R. Schmitz, P. D. Jeffrey, J. J. W. Wiltzius, P. Kussie, K. M. Ferguson, *Cancer Cell* **2005**, *7*, 301–311.
[12] C. C. Palsuledesai, M. D. Distefano, *ACS Chem. Biol.* **2015**, *10*, 51–62.
[13] S. Ulrich, D. Boturyn, A. Marra, O. Renaudet, P. Dumy, *Chem. Eur. J.* **2014**, *20*, 34–41.
[14] S. C. Jeffrey, J. De Brabander, J. Miyamoto, P. D. Senter, *ACS Med. Chem. Lett.* **2010**, *1*, 277–280.
[15] B. M. Chung, S. M. Raja, R. J. Clubb, C. Tu, M. George, V. Band, H. Band, *BMC Cell Biol.* **2009**, *10*, 84.

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