Ethynylphosphonamidates for the Rapid and Cysteine-Selective Generation of Efficacious Antibody–Drug Conjugates

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Dedicated to Professor Sam Gellman on the occasion of his 60th birthday

Abstract: *Requirements for novel bioconjugation reactions for the synthesis of antibody–drug conjugates (ADCs) are exceptionally high, since conjugation selectivity as well as the stability and hydrophobicity of linkers and payloads drastically influence the performance and safety profile of the final product. We report Cys-selective ethynylphosphonamidates as new reagents for the rapid generation of efficacious ADCs from native non-engineered monoclonal antibodies through a simple one-pot reduction and alkylation. Ethynylphosphonamidates can be easily substituted with hydrophilic residues, giving rise to electrophilic labeling reagents with tunable solubility properties. We demonstrate that ethynylphosphonamidate-linked ADCs have excellent properties for next-generation antibody therapeutics in terms of serum stability and in vivo antitumor activity.*

Antibody conjugates consisting of a drug linked to a tumor-selective antibody, so called antibody–drug conjugates (ADCs), are an emerging class of targeted therapeutics.[1] While most of the ADCs in clinical development contain cytotoxic molecules, recent studies also include the treatment of infectious diseases with antibody–antibiotic conjugates (AACs).[2] ADCs are particular interesting for the treatment of cancer, since they combine the high potency of cytotoxic molecules with the tumor specificity of monoclonal antibodies. ADCs thus have the potential to significantly broaden the therapeutic window compared to standard chemotherapy.[1,3] Recent progress in clinical development include the approval of inotuzumab ozogamicin (Besponsa)[4] and the re-approval of gemtuzumab ozogamicin (Mylotarg).[5] Nevertheless, challenges remain, especially in improving the linkage between drug and antibody.[6] Commonly used linker systems face problems such as insufficient serum stability and undesired aggregation behavior, which limits the number of drug molecules linked to an antibody and leads to undesired off-target toxicity.[7]

Maleimides have become the prime linker reagents for the generation of ADCs, including two approved ADCs: trastuzumab emtansine (Kadcyla) and brentuximab vedotin (Adcetris).[8] Maleimides can be applied to either modify native IgG antibodies through interchain-disulfide reduction and alkylation[9] or to engineered antibodies through addition to an additionally incorporated cysteine (Thiomab technology).[7a] Nevertheless, one of the biggest drawbacks of maleimide linkages is their susceptibility towards retro-Michael additions, which leads to premature drug cleavage during circulation and reattachment to cysteine-containing proteins like albumin.[7a,10] Even though consequences arising from such payload transfer are not yet fully understood, it is anticipated that the anti-tumor efficacy might be lowered due to decreased drug delivery to targeted cells. Furthermore toxic side effects might occur.[11] Several compound classes have been developed to overcome this issue, including self-hydrolyzing maleimides[11] and structurally refined Michael-type acceptors such as carbonyl acrylic derivatives[12] or exocyclic maleimides.[13] All of these methods yield stable sulfhydryl adducts; however, synthetic incorporation of these electrophiles into functional molecules remains challenging.[14]

Undesired aggregation of ADCs is another challenge, since many drugs used in the context of ADCs are hydrophobic.[15] The addition of organic co-solvents to the conjugation mixture is commonly employed to enable the conjugation of hydrophobic drugs, which however may affect the structural integrity of the antibody.[16] Additionally, the hydrophobic nature of drugs increases the formation of

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high-molecular-weight species (HMWS) in the final product.[17] Those aggregates impair the pharmacokinetic profile and efficacy[18] of ADCs and often limit the drug-to-antibody ratio (DAR) to a maximum of 4.[19] To overcome this issue, hydrophilic polyethylene glycol (PEG) linkers have been developed that compensate for the lipophilic nature of the drug.[20] However, it has recently been shown that PEG can negatively affect pharmacokinetics when incorporated as a linear spacer between antibody and drug.[21] Increasing the solvent exposure of the drug most likely facilitates unspecific hydrophobic interactions. This unwanted effect has been successfully mitigated by side-chain attachment of the solubilizing polymer.[21]

Based on our recently reported phosphonamidite-based labelling method,[22] we applied ethynylphosphonamidates as a novel compound class for the generation of stable Cys-linked ADCs. We initiated our studies by conjugating the antimitotic agent Monomethyl auristatin F (MMAF)[23] to the Her2-targeting antibody trastuzumab by using phosphonamidate functionalized cathepsin B cleavable linker 4, which was synthesized based on previously published procedures for Fmoc-protected Val-Cit dipeptides[24] (Figure S1 in the Supporting Information). In a first proof-of-concept study, we conjugated 4 to trastuzumab following our previously established method by applying 10 equiv labeling reagent per Cys,[22] giving an average DAR of 4.6 (Figure 1a and Figure S2 in the Supporting Information). To validate the functionality of trastuzumab–4, it was evaluated in a Her2-based proliferation assay with two Her2-overexpressing cell lines (BT474 and SKBR3) as well as a Her2-negative cell line as a control

(MDAMB468).[25] Since trastuzumab alone exhibits antiproliferative potency, cell viability was measured via a sensitive, high-content assay to asses retained antibody functionality after exposure to the conjugation procedure. The antibody concentrations leading to half maximal growth inhibition (IC_{50}) decreased with trastuzumab–4 compared to trastuzumab alone by 81-fold, from 900 to 11 pM, for SKBR3 cells (Her2 ++) and by 42-fold, from 800 to 19 pM, for BT474 cells (Her2 +). An effect on the proliferation of the control cell line MDAMB468 was only observed at very high ADC concentrations ($IC_{50} > 100$ nM). Notably, trastuzumab–4 inhibits the proliferation of close to 100% of cell population for SKBR3 cells, while trastuzumab alone only inhibits up to 50%. As an additional control, trastuzumab was treated with 4 without prior disulfide reduction. Those constructs behaved in a similar manner to the non-modified antibodies, thus highlighting the high Cys-selectivity, efficient removal of excessive toxin, and gentle reaction conditions of our method (Figure 1b). We additionally validated our measured IC_{50} values in a standard cell viability assay and obtained similar IC_{50} growth inhibition constants for trastuzumab–4 (Figure S3 in the Supporting Information). The mode of action of MMAF is the destabilization of microtubules through inhibition of tubulin polymerization.[26] Along these lines, we observed by fluorescence microscopy a disturbance of tubulin organization in BT474 cells upon treatment with 0.3 nM trastuzumab–4 for 4 days in contrast to proper spindle formation in untreated mitotic control cells (Figure 1c and Figure S4 in the Supporting Information).

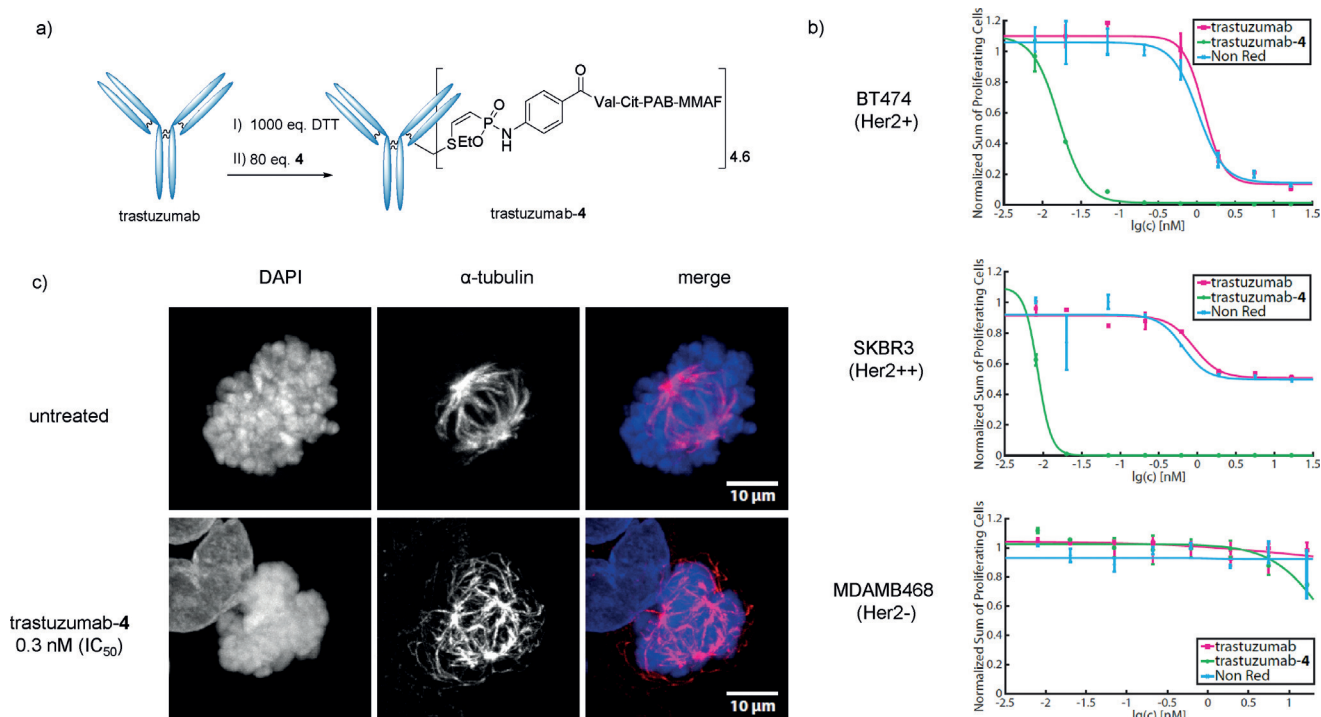*Figure 1.* a) Synthetic scheme for the attachment of 4 to trastuzumab. b) Antiproliferative potency of trastuzumab–4 on two Her2-overexpressing cell lines (SKBR3, BT474) and a control (MDAMB468). Plots depict the number of proliferating cells after 4 days of antibody treatment against antibody concentration. Non-Red-trastuzumab treated with 4 without prior disulfide reduction. c) Effect of trastuzumab–4 treatment on mitotic tubulin organization in BT474 (Her2 +) cells. Shown are representative images of mitotic BT474 cells after 4 days of treatment with 0.3 nM trastuzumab–4 compared to untreated cells.
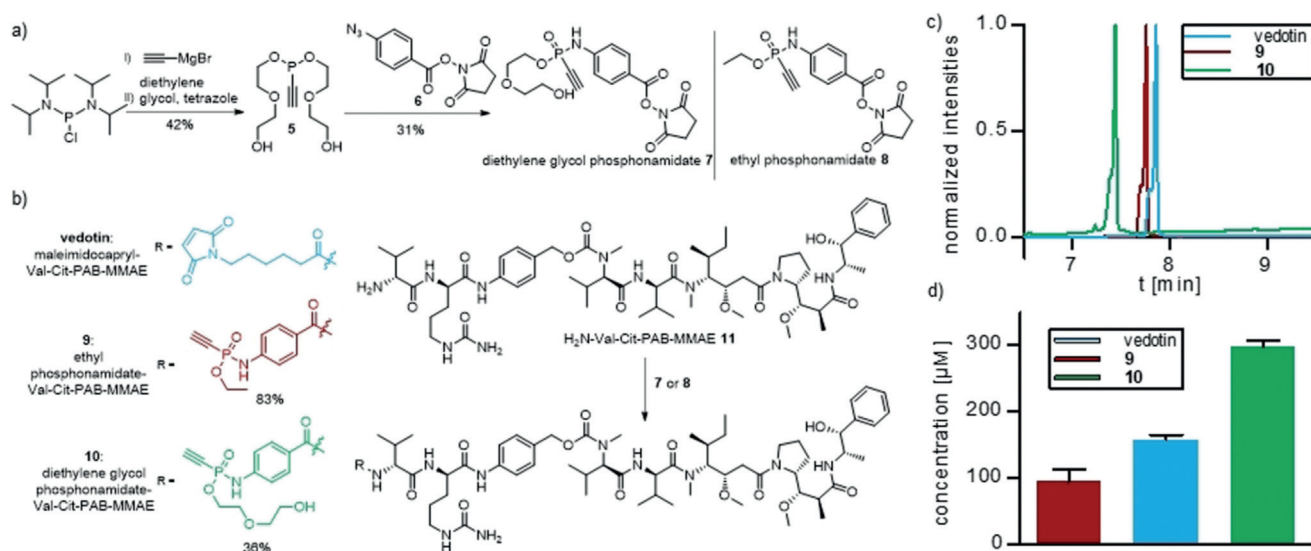
Figure 2. a) Synthesis of ethylene glycol substituted ethynylphosphonamidate building block **7** and structural comparison to the corresponding ethyl-substituted **8**.[22] b) Structure of vedotin and synthesis of ethyl (**9**) and diethylene glycol (**10**) phosphonamidate-based vedotin analogues. c) RP-HPLC analysis of vedotin and analogues **9** and **10**. d) Solubility in PBS with 5% DMSO of vedotin and analogues **9** and **10**. Error bars calculated from three independent measurements.

Furthermore, we anticipated that ethynylphosphonamidates bearing O-substituents with increased hydrophilicity might serve as powerful building blocks to increase the polarity of the whole linker system without increasing the overall linker length. Since the Staudinger-phosphonite reaction (SPhR) comprises a very convenient synthetic route to incorporate the ethynylphosphonamidate moiety into a given molecule, we focused on the synthesis of a hydrophilic phosphonite. Following our previous methods, we were able to synthesize the diethylene glycol substituted phosphonite **5** in a one-pot procedure.[27] Subsequent SPhR was performed with the NHS-modified azide **6** and yielded the desired diethyleneglycol-phosphonamidate **7** in 31% yield (Figure 2a).

To demonstrate the versatility of our method, we proceeded with the construction of a second antibody–drug pair. Since we wanted to directly compare our linkage technology with the maleimide linkage used in Adcetris, we continued our studies with phosphonamidate-linked ADCs that are structurally as close to Adcetris as possible. In addition to the MMAF constructs used in the previous study, two ethynylphosphonamidate MMAE derivatives were synthesized, one with an ethyl substituent at the phosphorous (**9**) and one with a diethylene glycol substituent (**10**; Figure 2b). RP-HPLC analysis showed a reduced retention time for compound **10**, when compared to **9** or vedotin (Figure 2c). Solubility measurements revealed that the aqueous solubility of the conjugates is drastically increased by the diethylene glycol substituent, from 95 μM (**9**) to 298 μM (**10**). The aqueous solubility of **10** is also twice as high as vedotin (Figure 2d and Figure S5 in the Supporting Information) Based on these observations we decided to proceed with the hydrophilic compound **10** for subsequent conjugation studies to antibodies.

Next, we tried to optimize our conjugation method to reduce the number of drug equivalents needed for sufficient conjugation. Since Adcetris is modified with an average of four vedotin molecules per antibody,[28] we started by screening different amounts of **10** to achieve similar modification and analyzed the DAR by intact protein MS (Figure S6 in the Supporting Information). We estimated that 16 equiv of **10** per antibody (2 equiv per Cys-residue) are needed to reach a DAR of 4 at 1 mg mL^{-1} antibody concentration. We attribute the required excess of phosphonamidate to slower reaction kinetics of ethynylphosphonamidates compared to maleimides.[22] To compensate for the slower kinetics, we increased the antibody concentration in the conjugation reaction to 5 mg mL^{-1}, a concentration that was previously also used for maleimide conjugations.[9] With this, we were able to use as little as 4.5 equiv of **10** per antibody to achieve a DAR of 4.0 (Figure S7 in the Supporting Information). After this, upscaling of the conjugation reaction to 2.4 mg brentuximab was performed at 1 or 5 mg mL^{-1} followed by a preparative size-exclusion chromatography step to ensure complete removal of the toxin prior to subsequent functional evaluations, yielding 1.6 mg of the desired ADC brentuximab–**10** with a DAR of 3.8–4.0. To furthermore simplify the conjugation process, we applied partial reduction of the interchain disulfide bonds with TCEP.[29] By screening TCEP equivalents needed to modify brentuximab, we found out that a partial reduction method with 3 equiv TCEP, 5 equiv **10**, and 5 mg mL^{-1} brentuximab, applied in a one pot process, produces an ADC with a DAR of 3.9 without the need for removal of reducing agent prior payload conjugation (Figure S8 in the Supporting Information). This one-pot process can be problematic with other cysteine-labelling reagents, since it has been shown that maleimides and vinyl sulfones for instance, irreversibly react with TCEP.[30]

Taking advantage of our ADC synthesis methods, we then evaluated commercially available Adcetris in comparison to our analogue brentuximab–**10** (Figure 3a). We started with a cell-based viability assay with a CD30-overexpressing cell
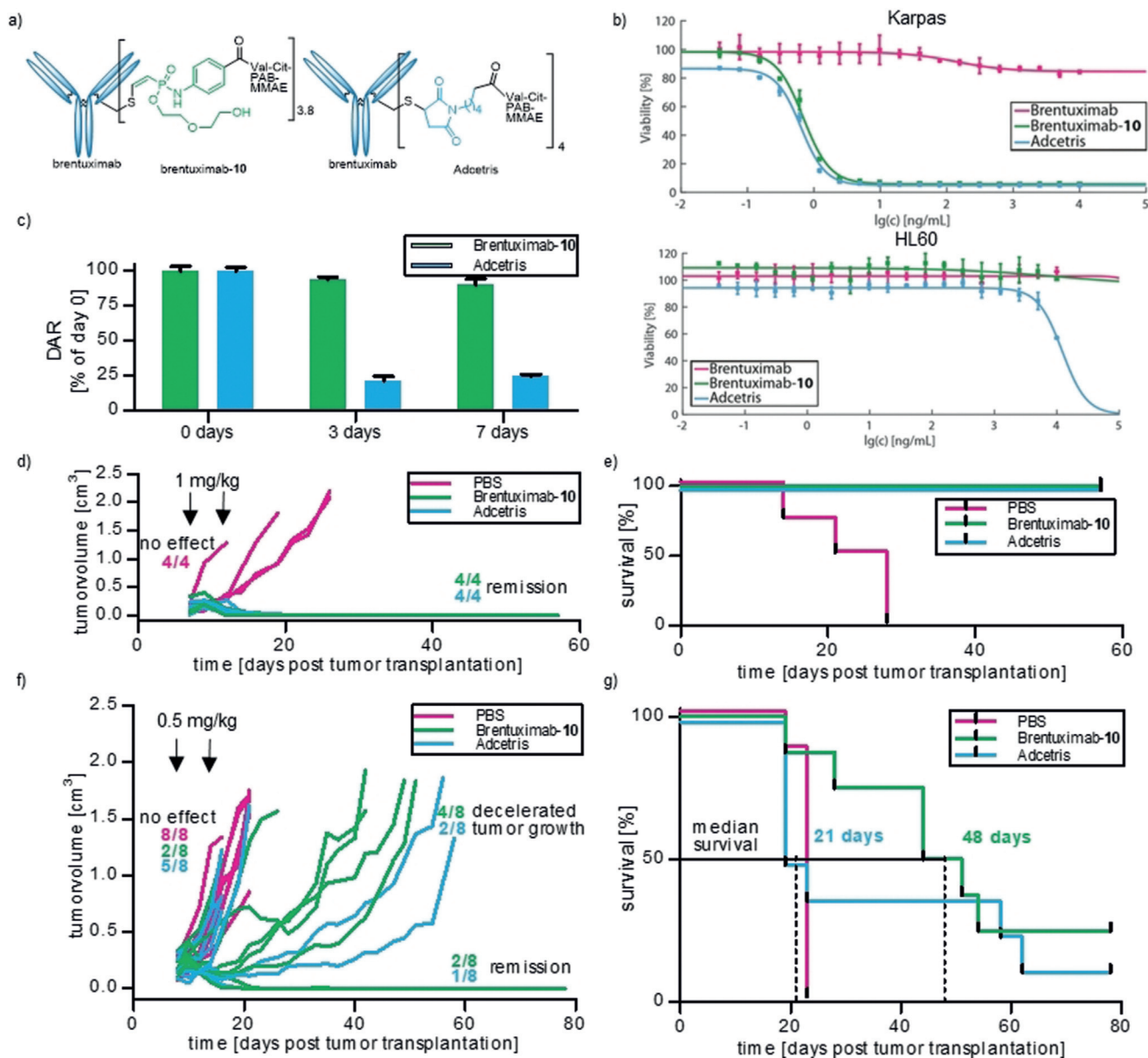
Figure 3. a) Structural comparison of Adcetris with brentuximab–**10**. b) Cell viability assays with a CD30-overexpressing cell line (Karpas299, top) and a control (HL60, bottom) for brentuximab–**10**, Adcetris· and brentuximab alone. c) Linkage-stability studies in rat serum. ADCs were incubated in rat serum for 0, 3, and 7 days at 37°C and analyzed by MS after pulldown, deglycosylation, and reduction. Shown is the DAR relative to the average DAR of day 0 for brentuximab–**10** and Adcetris. d) Antitumor activity of brentuximab–**10** and Adcetris and a PBS control in a Karpas 299 tumor xenograft model in SCID mice. Treatment of 1 mg kg^{-1} was administered twice at day 7 and day 10 after tumor transplantation. Tumor volumes of the four mice per group are shown separately. e) Kaplan–Meier survival analysis of the study shown in (d). f) Treatment of 0.5 mg kg^{-1} at day 8 and day 12 after tumor transplantation. Tumor volumes of the eight mice per group are shown separately. g) Kaplan–Meier survival analysis of the study shown in (f).

line Karpas299 and a CD30-negative cell line HL60 as a control. Both ADCs showed similar IC$_{50}$ growth inhibition constants in the range of 0.9 ng mL^{-1} in the antigen-positive cell line. Whereas Adcetris slightly affected the antigen-negative cell line at high ADC concentrations, we observed no such effect for brentuximab–**10**. (Figure 3b).

As mentioned earlier, increased stability of the linkage between drug and antibody in serum might improve the properties of ADCs in terms of off-target toxicity and anti-tumor efficacy.[11] In our experiment, we observed that 90% of

the phosphonamidate-linked MMAE was still connected to brentuximab after 7 days of incubation in rat serum at 37°C, as measured by intact-protein MS after pulldown of the ADC from serum. Under the same incubation conditions, Adcetris lost more than 70% of its payload after three days. (Figure 3c and Figure S9 in the supporting information). This data is in accordance with previous observations that describe a drastic DAR decrease of similar maleimide linked ADCs following 6 days of incubation in serum at 37°C.[31] Although hydrolysis of maleimides was shown to improve conjugate stability and

generates ADCs with increased in vivo potency,[11,32] the occurrence of incomplete hydrolysis of many maleimides may limit this approach.[33] Taken together, the stability experiments in serum clearly underlines our previously reported data on the excellent stability of the phosphonamidate linkage,[22] in particular when compared to maleimide-linked ADCs. Additionally, we performed storage tests with brentuximab–**10** to analyze the formation of HMWS, as reported previously.[34] Size-exclusion chromatography revealed less than 9 % HMWS formation with respect to the monomeric species after storage at 40 °C over two weeks. No significant increase in HMWS was observed after two weeks of storage at 4 °C (Figure S10 in the Supporting Information).

Finally, brentuximab–**10** was evaluated in vivo in a Karpas 299 derived tumor xenograft model in immunodeficient female CB17-SCID mice in a similar manner as previously reported.[35] In a first study, four mice were treated twice, at day 7 and day 10, with 1 mg ADC per kg bodyweight with brentuximab–**10**, Adcetris, or only PBS as a control. All 4 mice treated with brentuximab-**10** or Adcetris showed an excellent response to the treatment. Treated mice were in tumor remission already a few days after ADC injection and no relapse was observed over the whole observation period of 58 days, while the untreated control showed uncontrolled tumor growth and had to be sacrificed within three weeks after tumor transplantation (Figure 3d and Figure S11 in the Supporting Information). It was previously reported that an ADC with an average of four MMAE molecules connected to brentuximab via maleimides is significantly less efficacious in a tumor xenograft model when the dosing is lowered from 1 to 0.5 mg kg^{-1}.[35] From our serum stability studies (Figure 3c), we anticipated that phosphonamidite-linked ADCs might still be active at lower doses due to prolonged drug delivery in circulation. Therefore, we initiated a second in vivo study, with three groups of eight mice treated with either 0.5 mg kg^{-1} brentuximab–**10** or Adcetris or only PBS twice at day 8 and day 12. As expected, we observed decreased antitumor activity for both constructs when compared to the first study, leading to tumor remission in two mice for brentuximab–**10** and only one for Adcetris. However, five out of eight mice did not show any observable response to the treatment with Adcetris, and the tumor growth was as fast as in the PBS-treated control group. In contrast, this was only observed in two mice treated with brentuximab–**10** (Figure 3f and Figure S11 in the Supporting Information). Hence, we were able to show a drastic increase in median survival from 21 days for commercial Adcetris to 48 days for brentuximab–**10** (Figure 3g). This increase by a factor of 2.3 in comparison to Adcetris indicates promising antitumor activity for our novel phosphonamidate-linked ADCs. It should be noted that none of the mice, treated with either construct, showed significant changes in bodyweight over the observation period (Figure S11 in the Supporting Information).

In summary, we present ethynylphosphonamidates as cysteine-reactive handles for the construction of next-generation cancer therapeutics. By making use of the SPhR, we obtained a new modular diethyleneglycol-modified ethynylphosphonamidate building block for the synthesis of hydrophilic Cys-selective linker systems for the conjugation of unpolar payloads. With this method, we synthesized an ADC from brentuximab and MMAE, and demonstrated appropriate linkage stability combined with beneficial in vivo antitumor activity, resulting in an increased median survival from 21 days for Adcetris to 48 days for the phosphonamidate linkage. The conjugation method is straightforward, using only minimal drug excesses, and facilitates one-pot synthesis of ADCs starting from native antibodies. Taken together, the ethynylphosphonamidates described herein facilitate the straightforward construction of ADCs for cancer therapeutics and show great promise for other pharmacological targets.

Conflict of interest

The technology described in the manuscript is part of a pending patent application by M.-A.K., D.S., J.H., A.S, H.L. and C.P.R.H.

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