Highly Potent, Anthracycline-based Antibody–Drug Conjugates Generated by Enzymatic, Site-specific Conjugation

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Abstract

Antibody–drug conjugates (ADC) are highly potent and specific antitumor drugs, combining the specific targeting of mAbs with the potency of small-molecule toxic payloads. ADCs generated by conventional chemical conjugation yield heterogeneous mixtures with variable pharmacokinetics, stability, safety, and efficacy profiles. To address these issues, numerous site-specific conjugation technologies are currently being developed allowing the manufacturing of homogeneous ADCs with predetermined drug-to-antibody ratios. Here, we used sortase-mediated antibody conjugation (SMAC) technology to generate homogeneous ADCs based on a derivative of the highly potent anthracycline toxin PNU-159682 and a noncleavable peptide linker, using the anti-HER2 antibody trastuzumab (part of Kadcyla) and the anti-CD30 antibody cAC10 (part of Adcetris). Characterization of the resulting ADCs in vitro and in vivo showed that they were highly stable and exhibited potencies exceeding those of ADCs based on conventional tubulin-targeting payloads, such as Kadcyla and Adcetris. The data presented here suggest that such novel and highly potent ADC formats may help to increase the number of targets available to ADC approaches, by reducing the threshold levels of target expression required. Mol Cancer Ther; 16(5); 879–92. © 2017 AACR.

Introduction

Antibody–drug conjugates (ADC) take advantage of the specificity of mAbs for the targeted delivery of toxic payloads to cancer cells (1, 2). Because of the specific targeting of ADCs to the tumor, this class of drugs is expected to be associated with less side-effects and a higher therapeutic index than standard chemotherapy. Although highly plausible, this straightforward concept has only slowly translated into the clinic and the development of ADCs has been associated with difficulties and setbacks (1, 3).

The first ADC to be approved was Mylotarg (4), an anti-CD33 ADC for the treatment of acute myeloid leukemia (AML). This first-generation ADC suffered from unstable covalent coupling of the toxin to the antibody, leading to significant release of toxin molecules in circulation, which resulted in an unfavorable therapeutic index of this first-generation ADC. Eventually, Mylotarg, which had been FDA approved in 2000, was withdrawn from the U.S. market in 2010. Because of the safety concerns of this ADC, Mylotarg was never approved for cancer treatment by the European Medicines Agency (EMA; London, United Kingdom). More recently, also the anti-CD22 ADC inotuzumab–ozogamicin, comprising the same linker-payload as Mylotarg, yielded disappointing clinical data and did not show statistically significant benefit for the treatment of non-Hodgkin lymphoma (NHL; ref. 5).

As a result of the difficulties associated with such first-generation ADCs, the quality and functionality of chemical linkers for coupling toxins to antibodies has been improved substantially in recent years (6). Various new linker-payloads have been developed for incorporation into "second-generation" ADCs, including lysine-reactive noncleavable and cleavable derivatives of the maytansinoids, DM1 and DM4 (7), and cysteine-reactive derivatives of the auristatins monomethylauristatin F (MMAF) and E (MMAE; refs. 8, 9). Consequently, two ADCs, Adcetris and Kadcyla, have been approved for cancer therapy. Adcetris (brentuximab vedotin) is an ADC for the treatment of CD30-positive lymphomas (e.g., Hodgkin lymphoma), and employs a protease-cleavable linker based on the dipeptide valine–citrulline and a self-immolative p-aminobenzylcarbamate (PAB) group connected to the N-terminus of MMAE (8). Kadcyla (trastuzumab-emtansine or T-DM1) is an ADC approved for second-line therapy of HER2-positive breast cancer based on Herceptin, and employs a noncleavable SMCC thioether linker connected to DM1 (10).

Despite these highly encouraging developments, several challenges are still encountered even with second-generation ADCs. One challenge is related to the inherent instability of the utilized maleimide-containing linkers in serum. The thio–succinimid linkage present in these linkers can be reversed via a retro-Michael reaction involving free thiol groups present in high concentrations in human blood in the form of cysteine-34 of human serum albumin (11). This problem has been recognized and was recently addressed with the design of novel linker variants with maleimides displaying improved serum stability (12, 13).
The second challenge encountered by second-generation ADCs is related to their inherent heterogeneity. Conjugation of payloads to lysine or cysteine residues occurs randomly and only allows a limited control over the site of conjugation or the number of toxins coupled to the antibody (the drug-to-antibody ratio, or DAR; ref. 14). For the maytansine- and auristatin-based linker-payloads mentioned above, which are quite hydrophobic, it has been empirically determined that the “sweet spot” for the average DAR is between 3 and 4, which is also reflected by the DARs of the two commercial products, Adcetris and Kadcyla, which have been reported to be 4.0 and 3.5, respectively (15, 16). However, it is important to note that even with an average DAR in this range, the DAR is between 3 and 4, which is also re...

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>90% purity according to high-performance liquid chromatography (HPLC) analysis.

**Production of recombinant Sortase A variants from Staphylococcus aureus**

Highly active evolved Sortase A (eSrtA) was produced as described previously (24).

**Sortase-mediated toxin conjugation**

SMAC was produced essentially as described (24). Briefly, tagged mAbs [10 μmol/L] were incubated with oligo-glycine-modified toxin [200 μmol/L] in the presence of 3 μmol/L Sortase A in 50 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L CaCl₂, pH 7.5, for 3.5 hours at 25°C. The reaction was stopped by passing it through an rProtein A GraviTrap column (GE Healthcare) equilibrated with 25 mmol/L HEPES, 150 mmol/L NaCl, pH 7.5, followed by washing with 20 column volumes (CV) of buffer. Bound conjugate was eluted with 5 CVs of elution buffer (0.1 mol/L glycine, 50 mmol/L NaCl, pH 2.5) with 0.5 CV fractions collected into tubes containing 25% v/v 1 mol/L HEPES pH 8 followed by washing with 20 column volumes (CV) of buffer. Flow through and wash were collected in 0.5 CV fractions followed by washing with 20 column volumes (CV) of buffer. Bound conjugate was eluted with 5 CVs of elution buffer (0.1 mol/L glycine, 50 mmol/L NaCl, pH 2.5) with 0.5 CV fractions collected into tubes containing 25% v/v 1 mol/L HEPES pH 8 to neutralize the acid. Protein-containing fractions were pooled and formulated using ZebaSpin desalting columns (Thermo Fisher Scientific) according to the manufacturer's instructions. cAC10 ADCs were formulated in PBS without Ca²⁺ and Mg²⁺ (Amimed-Bioconcept) and Trastuzumab ADCs in Kadexyla-buffer (10 mmol/L sodium succinate pH 5.0, 175 mmol/L sucrose, 0.02% w/v polysorbate 20). ADCs used in in vivo studies were subjected to an additional polishing step prior to formulation by using 0.5 mL Pierce High-Capacity Endotoxin Removal Spin columns (Thermo Fisher Scientific) according to the manufacturer's instructions. For subsequent DAR 4.0 enrichment (Fig. 2) and the cytotoxicity assay described in Fig. 4A and B, TwinStrep-tagged trastuzumab was used for conjugation. Trastuzumab conjugates for DAR 4.0 enrichment were formulated in PBS.

**DAR 4.0 enrichment**

Tras-Gly₂-May and Tras-Gly₂-PNU (1.1 mg) produced as described above was passed over a 0.5 mL Streptactin Superflow high-capacity gravity flow column (IBA) and washed with PBS without Ca²⁺ and Mg²⁺. Flow through and wash were collected in fractions. Peak fractions as determined by Bradford assay were pooled and concentrated by ultrafiltration.

**ADC analytics**

The drug loading was assayed by reverse-phase chromatography performed on a PLRP-S 2.1 mm × 5 cm, 5 μm column (Agilent) run at 1 mL/min/80°C with a linear gradient between 0.05% TFA/H₂O and 0.04% TFA/CH₃CN. Prior to analysis, samples were reduced with DTT at 37°C for 15 minutes.

**Cell killing assays**

Cytotoxicity of ADCs was investigated in cell killing assays essentially as described previously (24). Briefly, cells were plated on 96-well plates in 75 μL growth medium and grown at 37°C in a humidified incubator in a 7.5% CO₂ atmosphere. The cell densities varied, depending on the experiment and cell lines (SKBR3 and T47D, 5,000 or 10,000 cells per well; Karpas-299, 2,500 or 5,000 cells per well; REH, 10,000 cells per well). After one day incubation, 25 μL of 3.5-fold serial dilutions of each ADC in growth medium were added, typically resulting in final ADC concentrations from 20 μg/mL to 0.02 ng/mL. Each dilution was done in duplicate. For competition, ADC serial dilutions were done in growth medium containing unconjugated trastuzumab at a concentration of 200 μg/mL. After 4 additional days, plates were removed from the incubator and equilibrated to room temperature. After approximately 30 minutes, 50 μL medium was removed carefully from each well and replaced with 50 μL CellTiter-Glo Luminescent Solution (Promega, catalog no. G7570). After shaking the plates at 450 rpm for 5 minutes followed by 10 minutes incubation in the dark without shaking, luminescence was measured on a Tecan Spark with an integration time of 1 second per well.

**In vitro serum stability**

To evaluate serum stability, cAC10-Gly₂-PNU was diluted to a concentration of 100 μg/mL in freshly prepared serum from Balb/c mice, or in commercially available sera from rat (Sigma), cynomolgus monkey (Abcam), and human (Sigma), and incubated at 37°C. Samples were removed and snap-frozen in liquid nitrogen on days 0, 3, 7, and 14 and stored at -80°C until ELISA analysis. Serum concentrations of mAb backbone and PNU-conjugated mAb were evaluated using an ELISA-based assay. For rodent sera, dilution series of ADC serum samples were captured on ELISA plates coated with 2 μg/mL of a mouse anti-PNU mAb (generated in-house by immunization of mice with Gly₄-EDA-PNU-conjugated ADC) to bind PNU-conjugated mAb, or with goat anti-human F(ab')² (Jackson ImmunoResearch) to bind the ADC's mAb backbone, and detected with a 1:2,500 dilution of an HRP-conjugated anti-human IgG (Jackson). For primate sera, dilution series of ADC serum samples were captured on ELISA plates coated with 2 μg/mL of recombinant extracellular domain of human CD30 (Sino Biological) and detected with a 1:2,500 dilution of HRP-conjugated donkey anti-human IgG (Jackson) to measure the concentration of the ADC's mAb backbone. To measure PNU-conjugated mAb, 2 μg/mL of mouse anti-PNU mAb followed by a 1:5,000 diluted HRP-conjugated goat anti-mouse F(ab')² (Jackson) was used for detection. Serum concentrations of PNU-conjugated mAb and total mAb were calculated from the half-maximal OD values of the sample titrations, by comparison with serial dilutions of a known concentration of the same ADC.

**Pharmacokinetic study**

Pharmacokinetics of cAC10 and cAC10-Gly₂-PNU was evaluated in female CD-1 mice. The choice of an immunocompetent mouse strain was based on our prior observation that low doses of antibody-based therapeutics in mice lacking endogenous antibodies suffer from a severely impaired half-life (Supplementary Fig. S1), possibly by rapid adsorption to unoccupied Fc receptors. Animals were treated with 1 mg/kg of unconjugated antibody or ADC by tail vein injection. Blood samples were collected at 1 hour, 24 hours, 72 hours, 7 days, and 14 days after injection. For each time point, three animals were bled. Individual animals were used for a maximum of two blood draws, at least one week apart. Blood was collected into tubes containing K₂-EDTA and plasma was isolated from blood by centrifugation at 1,500 × g for 10 minutes. Plasma was then transferred to sterile cryovials, aliquoted, and stored at -80°C until analysis. Plasma concentrations of mAb backbone and PNU-conjugated mAb was determined by ELISA, as described above. Noncompartmental pharmacokinetic parameters were calculated with Kinetica (Thermo Fisher Scientific).
In vivo efficacy studies

For the JIMT-1 xenograft model, 10^7 cells in 100 μL PBS/Matrigel (1:1) were implanted subcutaneously into the left flanks of CB17.SCID mice (Charles River Laboratories). For the Karpas-299 xenograft model, 5 × 10^6 cells in 100 μL PBS/Matrigel (1:1) were implanted subcutaneously into the left flanks of female NOD-SCID common-gamma chain-deficient (NSG) or CB17.SCID mice (Charles River Laboratories). In the following, primary tumor volumes were measured by a caliper. After mean tumor volumes had reached approximately 100 to 150 mm^3, tumor-bearing animals were block-randomized into treatment groups each according to tumor sizes. Group sizes were 5–6 animals in the case of NSG mice and 8 animals in the case of CB17.SCID mice. The next day (day 1), ADC therapy was initiated by intravenous injection of the indicated ADC. Treatment was repeated twice, in weekly intervals (i.e., on days 8 and 15). One day before each ADC administration (i.e., on days 0, 7 and 14), animals were pretreated by intravenous injection of 30 mg/kg IVIG (Privigen). Preconditioning of NSG and CB17.SCID mice with exogenous IgG was done because it was previously found to improve the intrinsically low half-life of mAbs and ADCs used at the low dose of 1 mg/kg in animals lacking endogenous antibodies (Supplementary Fig. S1). Animals that reached predetermined abortion criteria (tumor diameter 2 cm or 20% weight loss) during the course of the studies were sacrificed for ethical reasons. Tumor sizes and body weights were monitored for 43 days (JIMT-1), 69 days (Karpas-299 in NSG), or 57 days (Karpas-299 in CB17.SCID) after randomization.

Ethics statement

Animal experiments have been approved by the local Ethics Committee for Animal Experimentation. During the whole course of animal experiments, all efforts were made to minimize suffering.

Results

Generation of anthracycline-based linker-payloads for sortase A-mediated conjugation

The anthracyclines doxorubicin, nemorubicin, and PNU-159682 were evaluated for their suitability as ADC payloads. Doxorubicin is an anthracycline that is widely used as a chemotherapeutic agent for the treatment of various hematologic and solid tumors (36) and acts via DNA intercalation, although, the precise mechanism of action remains controversial (37). Nemorubicin, a morpholinyl analogue of doxorubicin, is significantly more potent than doxorubicin, also acts on multidrug-resistant cells and is not cardiotoxic at therapeutic doses (38, 39). PNU-159682 is a liver metabolite of nemorubicin and is about three orders of magnitude more potent than its parent molecule on cultured human tumor cells (34).

To allow for sortase-mediated conjugation, anthracycline derivatives were generated by adding a pentaglycine peptide to the tetracyclic aglycone structure, common to all anthracyclines. For this, the ^13C and the attached hydroxyl group were removed and the glycine stretch was added directly to the carboxyl group of ^13C via an amide bond using an ethylenediamine (EDA) linker, generating the linker-drugs Gly3-EDA-Dox, Gly3-EDA-Nemo and Gly3-EDA-PNU, respectively (Figs. 1A–C). In the case of PNU-159682, a cleavable linker-payload based on the protease-sensitive dipetide valine-citrulline (vc) and a self-immmolative spacer, PAB was also evaluated. For this, Gly3-vcPAB was attached to the primary alcohol of PNU-159682 through a N-formyl-N,N′-dimethylaminium spacer generating Gly3-vcPAB-PNU-159682 (Fig. 1D). A similar linker-payload for maleimide-based conjugation to thiols, referred to as NMS249, has recently been described and was evaluated in the context of a CD22-specific ADC (33).

Manufacturing of noncleavable, anthracycline-based ADCs targeting HER2

To allow for Sortase A-mediated conjugation, a variant of the HER2-specific therapeutic antibody trastuzumab was produced, with each of its C-termini tagged with an LPETG Sortase A recognition motif followed by a Strep-tag. In case of the heavy chain, the sequence was directly appended to the C-terminus. In case of the light chain, a five amino acid spacer peptide with the sequence GGGS was placed between C-terminus and tag. This spacer was previously shown to facilitate conjugation to the light chain, presumably by improving accessibility of the sortase A recognition motif for the enzyme (24). Functionally enhanced evolved sortase A (eStA; ref. 40) was then used to conjugate the respective Gly3-modified anthracycline-based linker-payload to the antibody. The transpeptidation reaction catalyzed by sortase A leads to the formation of a new peptide bond between the threonine residue of the LPETG sortase A recognition motif and the amino terminus of the linker-payload, therefore all resulting ADCs comprise the sequence LPETGGGGG in their linker. We previously showed that conjugation reactions with maytansine- and auristatin-based linker-payloads occurred with an efficiency of approximately 80–90%, yielding ADCs with DARs in the range of 3.1 to 3.5, which is similar to the DARs of the currently marketed ADCs, Kadcyla and Adcetris (24). Surprisingly, conjugation efficiencies with anthracycline-based linker-payloads were consistently and significantly higher and the ADCs Tras-Gly3-Dox, Tras-Gly3-Nemo, and Tras-Gly3-PNU manufactured here each had a DAR ranging between 3.7 and 3.9.

Enrichment of DAR 4.0 species

We next tested the feasibility of enriching DAR 4.0 conjugates, by taking advantage of the fact that sequences C-terminally appended to the sortase pentapeptide recognition motif are released upon successful conjugation. This unique feature of the sortase conjugation process should in principle allow the use of the Strep-tag as an affinity handle for the selective removal of under-conjugated ADC species by a simple Streptactin affinity chromatography step. Thus, a trastuzumab variant with a Twin-Strep-tag was produced and evaluated. Conjugation of Gly3-EDA-PNU to TwinStrep-tagged trastuzumab occurred nearly quantitatively on heavy and light chain and yielded a DAR of approx. 3.8, as shown by reverse-phase chromatography (Fig. 2A, left). Significantly, a Tras-Gly3-PNU conjugate polished by a single pass over a Streptactin affinity column did no longer show any traces of unconjugated heavy or light chain and was completely homogeneous with a DAR of 4.0 (Fig. 2A, right).

Selective removal of under-conjugated ADC species is of particular interest when using linker-payloads, which are less efficiently conjugated. Thus, the same strategy was used with a trastuzumab–maytansine conjugate. Whereas the initial ADC preparation was comparable with previously described maytansine-based conjugates (24) and exhibited a DAR of approx. 3.4 (Fig. 2B, left), a single pass over a Streptactin column removed the
majority of under-conjugated molecules to yield an almost homogeneous ADC preparation (Fig. 2B, right).

Taken together, these data demonstrate that SMAC technology allows efficient enrichment of DAR 4.0 ADCs. Nevertheless, given the very high conjugation efficiencies achieved consistently, especially with Gly5-anthracycline linker-payloads, all ADCs used in this study were evaluated without further purification.

**In vitro cell killing activity of noncleavable, anthracycline-based ADCs targeting HER2**

Next, the activity of SMAC technology–manufactured trastuzumab–anthracycline ADCs was evaluated in *in vitro* cell killing assays using the HER2-overexpressing breast cancer cell line SKBR3 (HER2high) and the HER2-negative acute lymphoblastic leukemia (ALL) cell line REH, as a negative control (Supplementary Fig. S2). HER2 expression levels in SKBR3 cells were determined experimentally using a bead-based assay (Biocytex) and found to be approximately 694,000 molecules per cell (Supplementary Fig. S3). Significantly, ADCs based on doxorubicin and nemorubicin showed hardly any toxicity and were only able to kill a minor fraction of HER2-overexpressing SKBR3 cells at high concentrations (Supplementary Fig. S2A). This low level of toxicity appeared to be specifically mediated by HER2, as no sign of toxicity was observed on HER2-negative cells (Supplementary Fig. S2B). In line with the dramatically increased cytotoxicity of the anthracycline PNU-159682 compared with other anthracylines (34), the Tras-Gly5-PNU ADC displayed a significantly enhanced cytotoxicity compared with the Tras-Gly5-Dox and Tras-Gly5-Nemo ADCs and killed SKBR3...
cells with an IC$_{50}$ of 2.8 ng/mL (Supplementary Fig. S2A). Also Tras-Gly$_3$-PNU-mediated cytotoxicity was highly specific, as only very high concentrations of the ADC had an effect on HER2-negative REH cells (Supplementary Fig. S2B).

In light of its high potency, the Tras-Gly$_5$-PNU conjugate was further investigated and compared with SMAC technology–manufactured trastuzumab-maytansine ADC (Tras-Gly$_3$-May), using HER2-overexpressing SKBR3 cells and the breast cancer cell line T47D, expressing low levels of HER2 (Figs. 3A and B). T47D cells were determined to express approximately 32,000 HER2 molecules per cell (Supplementary Fig. S3), that is, about 20 times less than SKBR3. Adcetris, which was used as a negative control, only had a modest effect on the growth of either cell line. As we had observed previously (24), Tras-Gly$_5$-May was highly effective on HER2$_{high}$ SKBR3 cells (Fig. 3A), with IC$_{50}$ of 18 ng/mL, but failed to have an appreciable cytotoxic effect on HER2$_{low}$ T47D cells (Fig. 3B). In striking contrast, Tras-Gly$_3$-PNU was effective on both cell lines, and killed SKBR3 as well as T47D cells with an IC$_{50}$ of 2.7 ng/mL and 14.7 ng/mL, respectively (Fig. 3A and B).

**Generation and evaluation of a noncleavable, PNU-based ADC targeting CD30**

To further validate SMAC technology–manufactured, PNU-based ADCs, the Gly$_2$-EDA-PNU linker-payload (Fig. 1C) was also conjugated to a sortase motif-modified version of the CD30-specific mAb cAC10, which is the basis of commercially available brentuximab vedotin (Adcetris; ref. 8). Activity of cAC10-Gly$_3$-PNU was evaluated and compared with Adcetris and to a maytansine-conjugated ADC, cAC10-Gly$_3$-May, in cytotoxicity assays, using the CD30$_{high}$ non-Hodgkin lymphoma (NHL) cell line Karpas-299 and the CD30-negative ALL cell line REH (Fig. 3C and D). Tras-Gly$_5$-May, which was used as a negative control, had no detectable growth-inhibitory effect on either cell line even at very high concentrations. In contrast, cAC10-Gly$_3$-PNU potently killed CD30$_{high}$ Karpas-299 cells, with an IC$_{50}$ of 1.8 ng/mL, and thus was more potent than Adcetris, which had an IC$_{50}$ of 4.8 ng/mL (Fig. 3C). cAC10-Gly$_3$-May showed a similar potency as Adcetris (IC$_{50}$ = 4.7 ng/mL). Significantly, none of the cAC10-based ADCs had any detectable effect on the growth of CD30-negative REH cells (Fig. 3D). Thus, these data demonstrate that our novel, noncleavable ADC format, based on sortase-mediated conjugation of a Gly$_2$-modified PNU-derivative, consistently delivers highly potent and specific drugs.

**Evaluation of PNU-based ADCs with a cleavable linker**

The noncleavable PNU ADC described above is likely to require lysosomal trafficking and degradation in late lysosomes for release of the payload to occur. We next evaluated whether the utilization of a cleavable linker would yield an even more potent ADC by increasing efficiency of payload release. Thus, a PNU-based linker-payload, incorporating the cathepsin B-cleavable dipeptide valine-citrulline (vc) and a self-immolative spacer, PAB (Fig. 1D), was conjugated to trastuzumab using SMAC technology. The cytotoxic activity of the resulting ADC, Tras-Gly$_3$-vcPAB-PNU, was then compared with that of the noncleavable variant, Tras-Gly$_5$-PNU, again using the breast cancer cell lines SKBR3 (HER2$_{high}$) and T47D (HER2$_{low}$), and the NHL cell line Karpas-299 (Figs. 4A and B; Supplementary Fig. S4). The CD30-specific ADC cAC10-Gly$_3$-PNU, which was used as a negative control, as expected was highly effective on HER2$_{high}$ SKBR3 cells (IC$_{50}$ = 3.7 ng/mL). Kadcyla, which was used as an additional control, as expected was highly effective on HER2$_{high}$ SKBR3 cells (IC$_{50}$ = 3.6 ng/mL), but did hardly exhibit a cytotoxic effect on HER2$_{low}$ T47D cells or on HER2-negative Karpas-299 cells. Importantly, use of a cleavable linker did not substantially increase ADC potency, and Tras-Gly$_3$-PNU and Tras-Gly$_3$-vcPAB-PNU exhibited cytotoxicity on SKBR3 cells with IC$_{50}$ values of 1.9 ng/mL and 0.24 ng/mL, respectively (Fig. 4A). The cytotoxic activity on HER2$_{low}$ T47D cells differed even less between Tras-Gly$_3$-PNU and Tras-Gly$_3$-vcPAB-PNU, with IC$_{50}$ values of 16.8 ng/mL and 12.0 ng/mL, respectively (Fig. 4B). Addition of a large excess...
Figure 3. \(\text{In vitro}\) cytotoxicity assays with Sortase A-conjugated noncleavable anti-HER2 and anti-CD30 ADCs. HER2-overexpressing SKBR3 breast cancer cells (A), HER2-low T47D breast cancer cells (B), CD30-high Karpas-299 NHL cells (C) and CD30-negative REH ALL cells (D) were grown in the presence of serial dilutions of the indicated ADCs. Viable cells were quantified using a luminescent cell viability assay. Datapoints represent mean of two replicates and error bars represent SD.
of unconjugated trastuzumab efficiently competed away most of the cytotoxic activity of both ADCs, indicating that the effect is specifically mediated via binding of the ADCs to HER2. However, these competition experiments also revealed that Tras-Gly3-vcPAB-PNU displayed a higher level of nonspecific cytotoxicity in comparison to its noncleavable counterpart, Tras-Gly5-PNU (Figs. 4A and B). Nonspecific cell killing activity of the cleavable ADC was also revealed on HER2-negative Karpas-299 cells, where Tras-Gly3-vcPAB-PNU was approximately 37 times more toxic than Tras-Gly5-PNU (Supplementary Fig. S4). As expected, addition of the large excess of unconjugated mAb. Viable cells were quantified using a luminescent cell viability assay. Datapoints represent mean of two replicates and error bars represent SD.

Figure 4. Comparison of in vitro cell killing activity of sortase A-conjugated anti-HER2 and anti-CD30 ADCs with cleavable and noncleavable linkers. HER2-overexpressing SKBR3 breast cancer cells (A), HER2-low T47D breast cancer cells (B), CD30-high Karpas-299 NHL cells (C) and CD30-negative REH ALL cells (D) were grown in the presence of serial dilutions of the indicated ADCs. Dotted lines, cell killing activity in the presence of an excess of unconjugated mAb. Viable cells were quantified using a luminescent cell viability assay. Datapoints represent mean of two replicates and error bars represent SD.
The cleavable Gly3-vcPAB-PNU-159682 linker-payload was also evaluated in the context of the CD30-specific mAb cAC10. The cytotoxic activity of the resulting ADC, cAC10-Gly3-vcPAB-PNU, was compared with that of the noncleavable variant, cAC10-Gly5-PNU, using the CD30high NHL cell line Karpas-299 and the CD30-negative ALL cell line REH (Figs. 4C and D). Tras-Gly5-PNU, which was used as a negative control, only had a modest effect on the growth of both cell lines. In contrast, cAC10-Gly5-PNU efficiently killed CD30-positive Karpas-299 cells (IC50 = 1.1 ng/mL), while having no appreciable effect on CD30-negative REH cells. Significantly, the cleavable cAC10-Gly3-vcPAB-PNU ADC did not show enhanced cytotoxicity on Karpas-299 cells (IC50 = 1.1 ng/mL). However, similar to the anti-HER2 ADCs, nonspecific cytotoxicity of the cleavable ADC on antigen-negative cells was increased significantly compared with its noncleavable counterpart (550-fold; Fig. 4D).

In summary, the results demonstrate that PNU-based ADCs manufactured by SMAC technology work efficiently in the absence of a cleavable linker and display enhanced nonspecific cytotoxicity in the presence of a cleavable linker.

**Figure 5.**
ADC serum stability (A–D) and pharmacokinetics of naked antibody and ADC (E). To determine in vitro serum stability, cAC10-Gly5-PNU conjugate was diluted in mouse (A), rat (B), cynomologus monkey (C) or human serum (D) and incubated at 37°C. Samples were collected at the indicated times and analyzed by ELISA to determine IgG and ADC concentrations. Solid lines, IgG; dotted lines, ADC. To measure pharmacokinetics of naked antibody and ADC, CD-1 mice were injected intravenously with 1 mg/kg cAC10 or cAC10-Gly5-PNU. Plasma samples were collected at the indicated times and analyzed by sandwich ELISA to determine IgG and ADC concentration (E).

**In vitro serum stability and pharmacokinetics of CD30-specific PNU conjugate**
Considering the high potency of noncleavable PNU-ADCs in vitro, and due to concerns about the stability and potential toxicity of cleavable PNU-ADCs in vivo, the focus of all additional experiments was on noncleavable PNU-ADCs. Thus, we next investigated the stability of the SMAC technology-manufactured cAC10-Gly5-PNU ADC in human serum and in serum from species relevant for preclinical efficacy and safety testing (Figs. 5A–D). For this, cAC10-Gly5-PNU was diluted in mouse, rat, cynomologus monkey, or human serum and incubated at 37°C for two weeks. Aliquots were removed at different times and the concentrations of total cAC10 antibody and intact ADC were determined by ELISA. The concentration of total cAC10 antibody remained essentially constant for the duration of the experiment. Significantly, also the concentration of intact ADC remained unchanged over time and payload loss could not be detected by toxin ELISA at any significant level. Thus, the novel noncleavable ADC format described herein, based on sortase-conjugation of a Gly5-modified PNU-derivative, is highly stable in serum from all species analyzed.
A pharmacokinetic study was carried out, to analyze in vivo half-life and stability of the cAC10-Gly5-PNU ADC in comparison to its parental mAb (Fig. 5E). For this, nontumor-bearing CD-1 mice were intravenously injected with cAC10-Gly5-PNU or naked mAb cAC10 as a control. Plasma was collected from the mice after various times and analyzed by ELISA to quantify intact ADC and total IgG. The pharmacokinetic parameters were determined by noncompartmental analysis. The time/concentration curves of cAC10 and cAC10-Gly5-PNU appeared to follow biexponential declines (Fig. 5E). The terminal half-lives of cAC10, cAC10-Gly5-PNU (total IgG detected by anti-Fc ELISA) and cAC10-Gly5-PNU (intact ADC detected by anti-PNU-toxin ELISA) were 13.1, 11.5, and 11.3 days, respectively. The half-life of nonconjugated cAC10 measured here thus correlates quite well with the 16.7 days reported previously (17). The half-life of cAC10-Gly5-PNU ADC was slightly reduced compared with the nonconjugated mAb cAC10. This is expected, because increased hydrophobicity of ADCs in comparison to nonconjugated antibodies is known lead to a faster turnover of the conjugate (17). However, comparison of intact ADC versus total IgG of the ADC revealed that there was hardly any detectable loss of PNU payload during the time of the experiment (14 days). Therefore, these data demonstrate that SMAC technology–manufactured PNU-ADCs are not only highly stable in vitro, but also in vivo.

In vivo antitumor activity of HER2-specific PNU conjugate

We next evaluated the in vivo efficacy of our novel SMAC technology–manufactured Tras-Gly5-PNU ADC in a trastuzumab-resistant JIMT-1 breast cancer xenograft model. JIMT-1 cells are known to express moderate HER2 levels despite gene expression in vitro, allowing to address targets expressed at levels not accessible to ADCs with conventional payloads.

In vivo antitumor activity of CD30-specific PNU conjugate

We then evaluated the in vivo efficacy of the SMAC technology–manufactured cAC10-Gly5-PNU ADC in two different CD30-positive NHL mouse xenograft models in comparison to: (i) Adcetris, as a benchmark control; (ii) SMAC technology–manufactured Tras-Gly5-PNU ADC, as a negative control; and (iii) SMAC technology–manufactured cAC10-Gly5-Maytansine ADC, as an additional control (Figs. 6B and C). For this, CD30high Karpas-299 cells were either transplanted subcutaneously into NSG, or into CB17.SCID mice. In both models, tumors grew very aggressively in vehicle control groups and reached sizes of around 1,000 mm³ within 10 or 14 days post randomization, respectively. Mice were treated intravenously 3 times weekly with different ADC preparations, beginning one day post randomization, when the tumors had reached a size ranging between 100 and 150 mm³. Interestingly, Adcetris treatment in Karpas-299 xenotransplanted NSG mice showed only limited efficacy at the standard concentration of 1 mg/kg and tumor growth was only delayed by about a week (Fig. 6B). In line with the previously reported high efficiency of Adcetris in a Karpas-299 model in SCID mice (8), Adcetris was highly effective in the CB17.SCID model at a dose of 1 mg/kg, leading to tumor regression in all treated animals (Fig. 6C). In contrast to Adcetris, SMAC technology–manufactured anti-CD30 ADCs (either with Gly5-PNU at 1 mg/kg or with Gly5-Maytansine payload at 10 mg/kg) were found to be highly effective for the treatment of Karpas-299 tumors in both models, although also these ADCs were slightly less efficacious in NSG mice (Figs. 6B and C). As expected, animals treated with Tras-Gly5-PNU ADC as a negative control developed tumors with similar kinetics as in the vehicle control groups in both models, showing that the effect of the cAC10-Gly5-PNU and cAC10-Gly5-May ADCs was highly antibody and target specific.

Significant differences in the efficacies of the different ADC preparations become apparent when antitumor effects are analyzed in individual mice (Supplementary Fig. S6). In the NSG model, all mice treated with cAC10-Gly5-May, which initially responded well to the 10 mg/kg treatment, eventually relapsed. Thus, in this model antitumor activity of the maytansine conjugate appears to require the continuous administration of the ADC to permanently suppress tumor growth. In striking contrast, even in NSG mice, treatment with cAC10-Gly5-PNU at the much lower dose of 1 mg/kg led to a long lasting tumor regression until day 69 in all but one of the animals.

These data demonstrate that SMAC technology–manufactured anti-CD30 ADCs exhibit comparable or, depending on the preclinical animal model employed, even better potency than the approved ADC Adcetris. Most notably, only the SMAC technology–manufactured cAC10-Gly5-PNU ADC leads to long-lasting tumor eradication in a majority of animals in both models, even without continuous ADC administration.

Significantly, all ADCs were welltolerated, as shown by the absence of weight loss in each of the treatment groups compared with the vehicle control group (Supplementary Fig. S5B and S5C).

Discussion

Numerous strategies to improve properties of conventional ADCs have been described in recent years, aiming at increasing the degree of homogeneity, level of serum stability and/or cell
killing potency (1, 2). In this study, we describe an ADC platform based on SMAC technology (24), which addresses all these aspects. The ADCs produced are homogeneous by way of their predefined attachment sites for the payload on the C-termini of the antibody’s heavy and light chains. The use of a noncleavable, entirely peptide-based linker confers exquisite serum stability, whereas a highly potent payload derived from the anthracycline PNU-159682 provides superior potency. We have previously described the use of SMAC technology for the generation of maytansine- and auristatin-based ADCs (24). Enzymatic conjugation using an engineered sortase A enzyme from S. aureus allowed site-specific attachment of pentaglycine-modified payloads with an efficacy of 80% to 90%. This correlates well with the conjugation efficiency described in this study, where a trastuzumab–maytansine conjugate with a DAR of approx. 3.4 was generated (Fig. 2B), corresponding to a conjugation efficiency of 85%. As shown in this study, the use of a StrepTactin affinity chromatography step allows for depletion of underconjugated ADC species and manufacturing of homogeneous DAR4.0 ADCs. Surprisingly, we found that the Gly5-PNU linker-payload allowed for significantly better conjugation efficiencies than other payloads, with efficiencies often exceeding 95% and DARs close to 4.0 routinely achieved without any additional enrichment step (Fig. 2A). It is conceivable that accessibility of the oligo-glycine peptide for the enzyme’s active site differs between various Gly5-modified payloads, leading to different conjugation efficiencies. The high degree of compatibility of the Gly5-PNU linker-payload with the sortase enzyme allowed us to generate and analyze high-quality ADCs without further purification.

Anthracyclines such as doxorubicin, epirubicin, and daunorubicin are frequently used chemotherapeutic agents for the...
treatment of hematologic and solid cancers (42). Accordingly, a doxorubicin-based ADC has already been evaluated for cancer-cell targeting many years ago (43). While this ADC, like other early day ADCs based on chemotherapy agents, suffered from a number of issues related to immunogenicity and linker instability, also the potency of the standard chemotherapeutic agents used as payloads was simply too low for targeted delivery (2). Indeed, a subsequently evaluated ADC called BR96-doxorubicin, targeting the Lewis Y antigen, successfully circumvented the hypersensitivity reactions by using a mouse/human chimeric mAb but did not progress beyond phase II due to insufficient efficacy (44). It will be interesting to see how Immunomedics’s anti-CD74 conjugate milatuzumab–doxorubicin for the treatment of multiple myeloma performs in the clinic (http://www.immunomedics.com/adc.shtml). In line with clinical results available to date, the ADCs utilizing linker-payloads based on doxorubicin or nornorubicin analyzed in this study did not display significant cell killing activity (Supplementary Fig. S2A).

In light of the limited potency of doxorubicin- and nornorubicin-based ADCs, the Gly5-PNU linker payload, based on a derivative of the nornorubicin metabolite PNU-159682, was also investigated in the context of SMAC technology. PNU-159682 was described to be several orders of magnitude more potent than doxorubicin (34) and has recently shown promise as a payload for ADCs in the context of conventional chemical conjugation (32, 33). Indeed, when conjugated to trastuzumab, a significantly increased cytotoxic activity was achieved in comparison with maytansine conjugates and even treatment of a significantly higher fraction of metastatic breast cancers, as well as several additional types of cancer. Considering that HER2 is widely expressed in numerous healthy tissues (46), it will be interesting to see the results of the clinical evaluation of such high potency anti-HER2 ADCs.

An additional advantage of highly potent ADC formats such as the one described here is that they may be effective at relatively low target receptor densities, thereby increasing the number of targets accessible to ADC strategies. The current paradigm demands that the ideal ADC target be not only tumor-specific, but also highly expressed and/or highly internalizing (2, 3). It is reasonable to expect that there are numerous potential ADC targets that fulfill the requirement for tumor-selective expression, but fail to show sufficient expression levels and/or sufficient internalization rates for conventional ADCs. Time will tell how the opportunity provided by high potency ADCs, as described in this study, will translate into the clinic.

In light of the highly potent PNU payload evaluated in this study, we decided to use a noncleavable pentaglycine linker in our initial trastuzumab- and cAC10-based ADCs. Noncleavable linkers provide the highest degree of in vivo stability (6) and therefore have the potential to reduce nonspecific toxicity of highly potent ADCs. Significantly, we demonstrated that noncleavable ADCs based on the PNU payload were not only highly potent, but also effective on cells expressing only moderate target levels (Figs. 3C and D). Nevertheless, we wondered whether the use of a cleavable linker would further enhance the potency of PNU-based ADCs. Efficient delivery of a toxic payload is commonly believed to be improved by using cleavable linkers. Whereas early hydrazine-based linkers suffered from insufficient stability, more recent cleavable linkers, such as reducible disulfide-based or enzymatically cleavable di-peptide linkers (7, 8), achieve a high level of in vivo stability and are by far the most frequently used linkers in clinical-stage ADCs (2). Surprisingly, only a moderate improvement of the cytotoxic activity of trastuzumab-based ADCs was achieved when using a cleavable vcPAB linker, especially on target cells expressing low target levels (Figs. 4A and B). In the case of cAC10-based ADCs, use of a cleavable linker had no appreciable effect at all (Fig. 4C), indicating that efficient release of the PNU payload inside cells occurs even in the absence of a cleavable linker. In addition, the nonspecific cell killing activity of cleavable PNU-based ADCs was found to be significantly higher, as shown by competition experiments with unconjugated mAb (Figs. 4A and B), and by evaluation of target-negative cells (Supplementary Fig. 5A and Fig. 4D). Thus, at least in the context of SMAC technology, a noncleavable linker appears to be the preferred choice for a PNU-based ADC, by combining high efficacy with increased selectivity.

The ADC platform described here is universally applicable. We show that in addition to maytansines and auristatins (24), also anthraycines can be modified with oligo-glycine linkers and efficiently conjugated using SMAC technology. Furthermore, the Gly5-PNU payload described here can be attached to different mAbs and consistently delivers highly potent ADCs. For instance, conjugation of Gly5-PNU to the CD30-specific mAb and an auristatin payload (http://www.mersana.com/pipeline). The fact that numerous highly potent anti-HER2 ADCs are currently being developed underlines the attractiveness of HER2 as a cancer target. The ability to address not only cancer cells displaying extremely high levels of HER2 (IHC status 3+) but also those expressing moderate levels (IHC status 1+ or 2+) has the potential to allow treatment of a significantly larger fraction of metastatic breast cancers, as well as several additional types of cancer. Considering that HER2 is widely expressed in numerous healthy tissues (46), it will be interesting to see the results of the clinical evaluation of such high potency anti-HER2 ADCs.

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However, we found a striking difference in the antitumor efficacy of the clinically approved benchmark ADC Adcetris in Karpas-299 xenograft models, depending on the mouse strain used. Whereas antitumor activity was limited in NSG mice, Adcetris was very potent on Karpas-299 tumors in CB17.SCID mice (Fig. 6). Similar strain-specific differences in the efficacy of cAC10-MMAE conjugates have been observed recently and were attributed to significantly faster clearance of the ADCs in NSG versus SCID mice (13). Interestingly, this observation was not limited to specific ADCs or ADC formats, but rather a general feature of mAb-based drugs including naked mAbs. Although we have not performed a pharmacokinetic study in mice, our initial trastuzumab- and cAC10-based ADCs lead to permanent eradication in CB17.SCID, but not in NSG host mice, where only a transient antitumor effect was observed. The fact that our SMAC technology–manufactured cAC10-Gly5-PNU-ADC maintains a strong antitumor activity...
even in NSG mice is an impressive demonstration of the high potency of PNU-based, SMAC technology–conjugated ADCs.

The site of conjugation has previously been shown to have a significant impact on in vivo stability, thereby influencing the toxicity profile and antitumor efficacy of an ADC (23, 47, 48).

With respect to the placement of payloads at the C-termini of an antibody's heavy or light chains, there have been conflicting results. Suboptimal serum stability has been observed when using cleavablevcPAB linkers (47). However, this observation seems to be specific to this type of cleavable linker and can be mitigated by changes in the linker design (49).

Accordingly, no stability issues have been observed upon C-terminal conjugation using noncleavable linkers (23, 30). In agreement with these observations, the C-terminally conjugated ADCs described here, employing noncleavable peptide linkers, are highly stable in serum, both in vitro (Figs. 5A–D) and in vivo (Fig. 5E). The avoidance of maleimide-based linkers, shown to be associated with a certain degree of serum instability of conventional ADCs (11), is likely to add to the stability of ADCs generated using sortase.

In summary, our results show that SMAC technology is capable of generating highly homogeneous, stable and potent ADCs based on a derivative of the anthracycline PNU-159682, that can result in long-lasting and complete tumor eradication with only 3 treatment cycles in xenotransplantation models. It will be interesting to see how these early preclinical data will translate into the clinic. First clinical evaluation of this novel ADC format is expected in 2019.

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Disclosure of Potential Conflicts of Interest

R.R. Beerli and U. Grawunder hold stocks of NBE-Therapeutics AG. This work has been included in a patent application by NBE-Therapeutics AG. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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Development of methodology: N. Stefan, U. Grawunder, R.R. Beerli

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Stefan, R. Gebieux, L. Waldmeier, T. Hell, M. Escher, F.J. Woler.

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Stefan, L. Waldmeier, R. R. Beerli

Writing, review, and/or revision of the manuscript: N. Stefan, R. Gebieux, U. Grawunder, R.R. Beerli

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Stefan

Study supervision: N. Stefan, U. Grawunder, R.R. Beerli

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891

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Highly Potent, Anthracycline-based Antibody–Drug Conjugates Generated by Enzymatic, Site-specific Conjugation

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