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Site-Specific Traceless Coupling of Potent Cytotoxic Drugs to Recombinant Antibodies for Pharmacodelivery

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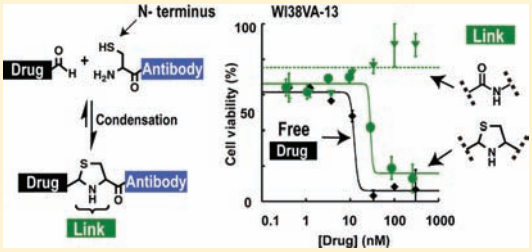
Supporting Information

ABSTRACT: Aldehyde drugs are gaining increasing research interest, considering that aldehyde dehydrogenases overexpression is characteristic of cancer stem cells. Here, we describe the traceless site-specific coupling of a novel potent drug, containing an aldehyde moiety, to recombinant antibodies, which were engineered to display a cysteine residue at their N-terminus, or a 1,2-aminothiol at their C-terminus. The resulting chemically defined antibody-drug conjugates represent the first example in which a thiazolidine linkage is used for the targeted delivery and release of cytotoxic agents.



INTRODUCTION

The selective chemical modification of proteins represents an important avenue for the study of protein function and for the development of novel biopharmaceuticals.[1,2] In particular, bio-orthogonal reactions, which modify proteins at specific sites using cleavable linkers, are essential for the development of antibody-drug conjugates (ADCs).[3−5] This emerging class of biopharmaceuticals consists of armed versions of therapeutic antibodies, which mediate the selective delivery and release of potent cytotoxic agents at sites of disease, sparing healthy tissues. In spite of the large variety of bio-orthogonal reactions available, most of the current ADCs manufacturing processes still exploit the chemical modification of the primary amino group of lysine or of the thiol group of cysteine residues,[6,7] thus, yielding heterogeneous mixtures, with possible potency loss and batch-to-batch reproducibility problems.

Ketones and aldehydes have received particular attention for the implementation of innovative bio-orthogonal protein modification strategies. These electrophiles readily condense with weak amine nucleophiles whose reactivity is enhanced by the α-effect,[8] while being poorly reactive in the presence of other biological nucleophiles (e.g., amines, thiols, and alcohols). Recent elegant studies reported the genetic encoding of an aldehyde tag into proteins, allowing their site selective modification with hydroxylamines[9] or the formation of hydrazones for the selective capture and release of proteins from complex biological mixtures.[10] The condensation between carbonyls and 1,2- aminothiols at pH 4−5 to yield thiazolidine heterocycles (Figure 1a) represents a bio-orthogonal reaction[8] that has been used in convergent protein synthesis and covalent capture techniques,[11] but not with biosynthetically produced proteins and for ADC development. In proteins, a cysteine residue in N-terminal position represents a natural source of 1,2-aminothiol functionality; however, protein biosynthesis incorporates methionine as first amino acid. Methods for producing recombinant proteins with an N-terminal cysteine residue all rely on cleavage of an appropriate upstream amino acid sequence.[12,13] Recently, 1,2-aminothiols have been genetically encoded into recombinant

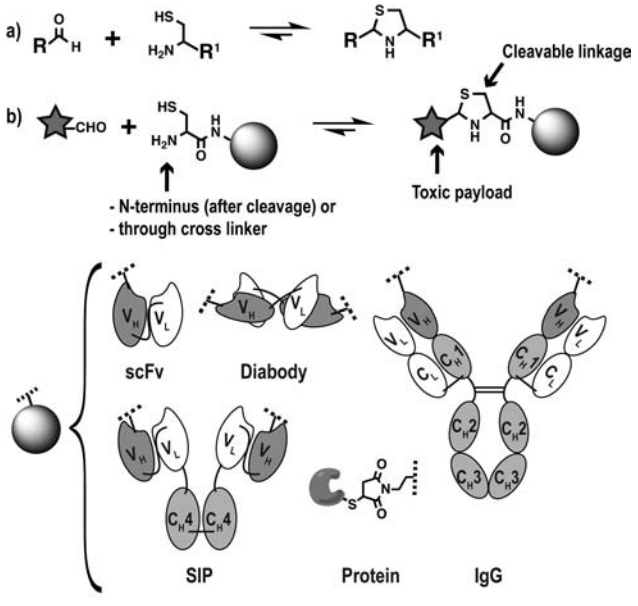


Figure 1. (a) Thiazolidine heterocycle forming reaction. (b) An aldehyde drug undergoes thiazolidine formation by reaction with a 1,2-aminothiol functional group introduced in a protein either by engineering of the N-terminus or by the use of a suitable cross-linker. Relevant antibody formats are also depicted, together with a protein modified using a maleimido-based bifunctional linker.

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proteins and used for site-specific protein labeling.[14] We reasoned that introducing a cysteine immediately after the leader peptide sequence in mammalian cell production would afford an N-terminal cysteine upon protein secretion. The newly introduced 1,2-aminothiol would be compatible with highly selective protein modification strategies, such as native chemical ligation,[15] cyanobenzothiazole condensation,[14] or the coupling and release of potent aldehyde drugs. Indeed, thiazolidine formation would represent a general approach for the synthesis of a new generation of ADCs, which would release both the aldehyde drug and the original antibody in a scarless manner (Figure 1).

METHODS

Mammalian Cell Culture and Antibody Production. Antibodies were expressed using transient gene expression (TGE).[16] Cells were handled under sterile conditions in a laminar air flow hood using disposable sterile plastic pipettes (TPP), sterile pipet tips, and tissue culture flasks of different sizes (TPP). Incubation of cells occurred either in a humidified incubator (series II Water Jacketed CO_2 Incubator, ThermoForma) with a 5% CO_2/95% air atmosphere or in a shaking incubator (Innova 4000 or 4430) at 180 rpm (only suspension cells). The temperature for protein production was 31 °C and for cell growth, 37 °C. The plasmids containing the correct construct were used to transfect Chinese Hamster Ovary cells (CHO-cells): 1.25 μg of DNA was used to transfect 1 mllion cells. Polyethyleneimine (PEI) was used to help DNA in the phagocytosis. A total of 50 μg/mL DNA and 250 μg/mL PEI were mixed and incubated at room temperature for 10 min. Subsequently, the DNA–PEI complex was added to the cells, previously resuspended in proCHO-4 medium at a concentration of 2 million cells/mL, and gently mixed. The transfected cultures were then incubated at 37 °C for 4 h in a shaking incubator (160 rpm), diluted with the same volume of powerCHO-2 CD medium, and then incubated for 6 days at 31 °C in a shaking incubator (140 rpm). The antibodies were subsequently purified from the cell culture by protein A affinity chromatography (GE Healthcare, n Protein A, sepharose 4 fast flow, 17-5280-04), after an initial passage on a sepharose resin (PD10 column, GE Healthcare 17-0851-01) to remove unspecific resin binders and cellular debris. The protein A column was washed with phosphate-buffered saline (PBS) pH 7.4, then with 0.1 M NaCl in PBS pH 7.4, containing 0.1% Tween and finally again with PBS pH 7.4. Proteins were eluted with 0.1 M glycine, pH 3, adjusting the pH immediately after elution by addition of 1/10 of the volume of 1 M Tris-HCl, pH = 7. The optical density (OD) of the different fractions was measured at 280 nm (Cary300 UV–visible spectrophotometer, Varian) and all fractions with an OD_{280} higher than 0.1 were pooled. The combined fractions were dialyzed at 4 °C against PBS using SpectraPor dialysis membranes (12–14 kDa MWCO). Typical nonoptimized antibody yields were 5 mg/L of culture.

N-Terminal Cysteine Modified Antibody Pretreatment. After purification, antibodies were first reduced in PBS with 1,4-dithiothreitol (DTT) (10 mM, 1 h) and subsequently treated with 4 M methoxyamine hydrochloride ($MeONH_2$·HCl) (final concentration 400 mM, pH 3–4, 16 h). The mixture was purified by fast protein liquid chromatography (FPLC) (Amersham Pharmacia) using two HiTrap desalting columns in series (GE Healthcare) of 5 mL volume equilibrated with potassium acetate buffer (AcOK, 100 mM, pH 4.5, for thiazolidines condensation) or with PBS (for native chemical ligation experiments, see Supporting Information (SI)) at a flow rate of 1 mL/min. The fractions containing the antibody were collected, concentrated, and analyzed using liquid chromatography–mass spectrometry (LC–MS) and sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). ESI–MS: Cys-Db(F8) (calcd 24890.0 Da, found 24890.0 Da, Figure 2e); Cys-Gly-Db(F8) (calcd 24947.0 Da, found 24946.0 Da, Figure 2h).

1,2-Aminothiol Functional Group Introduced with a Cross-Linker. The F8 antibody in diabody format, carrying a cysteine residue at the C-terminus (Db(F8)-Cys),[17] dissolved in PBS (29 μM, 0.058 μmol, 0.73 mg/mL, 2 mL), was reduced with tris(2-carbox-yethyl)phosphine hydrochloride (TCEP·HCl) (20 equiv, 11.6 μL from 100 mM solution in

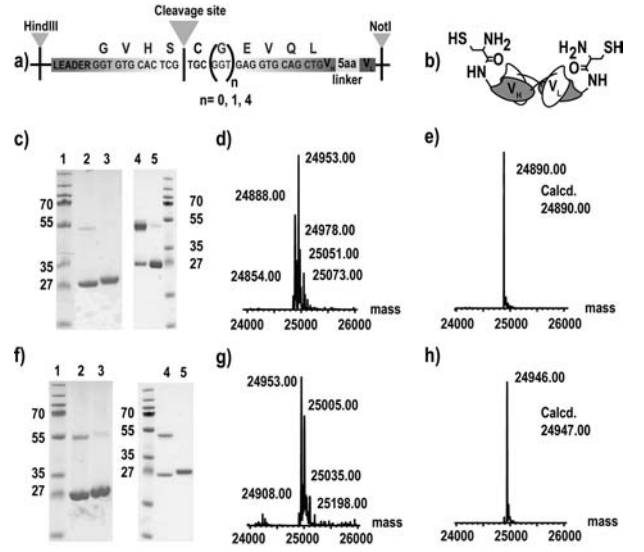

Figure 2. (a) DNA structure and amino acid sequence of an engineered F8 antibody mutant, carrying a Cys residue (**C**) immediately after the last residue of the leader peptide sequence used for protein secretion (**S**). The exact position of the cleavage site is shown. Three different mutants were cloned, containing 0, 1, or 4 Gly residues after the Cys residue. (b) Schematic representation of a recombinant antibody in diabody format (Cys-Db), carrying a Cys residue at the N-terminus of the V_H domain (gray), after cleavage of the leader peptide. The diabody format consists of a V_H domain sequentially linked to the V_L domain (white) of the same antibody by means of a short (n = 5) peptide linker, which drives the noncovalent, yet stable, homodimerization of two V_H–V_L polypeptides. In the Cys-Db(F8) variants, the N-terminal Cys residue is depicted in order to illustrate the site for selective thiazolidine formation. (c) SDS–PAGE analysis of Cys-Db(F8); lanes 2–3, Cys-Db(F8) in nonreducing and reducing conditions;[17] lanes 4–5, Cys-Db(F8) after pretreatment with DTT and $MeONH_2$·HCl (nonreducing and reducing conditions). (d and e) ESI–MS of Cys-Db(F8) immediately after purification on Protein A resin and after pretreatment with DTT and $MeONH_2$·HCl. (f) SDS–PAGE and (g and h) ESI–MS analysis of Cys-Gly-Db(F8), in similar conditions as described for the Cys-Db(F8) mutant.

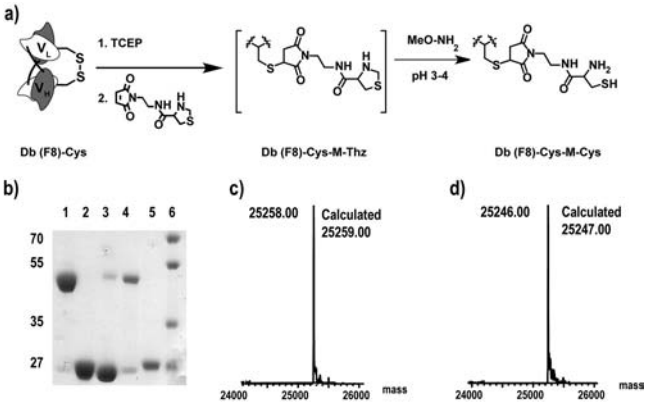

Figure 3. (a) Synthetic scheme for the site-selective 1,2-aminothiol incorporation at a C-terminal cysteine residue in the diabody Db(F8)-Cys. (b) SDS–PAGE monitoring of the Db(F8)-Cys modification process; lanes 1–2, Db(F8)-Cys in nonreducing and reducing conditions; lane 3, Db(F8)-Cys-M-thz, a reaction intermediate run under nonreducing conditions; lanes 4–5, Db(F8)-Cys-M-Cys (nonreducing and reducing conditions). (c and d) ESI–MS of reduced Db(F8)-Cys-M-thz and Db(F8)-Cys-M-Cys, respectively.

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water, 4 °C, 9 h, Figure 3). To that mixture, *N*-(2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethyl)thiazolidine-4-carboxamide (40 equiv, 23 μL from 100 mM solution in water) was added and incubated at 4 °C. After 15 h, the excess of the maleimido reagent was quenched with β-mercaptoethanol (16 μL, 100 equiv with respect to the maleimide reagent) during 1 h at 4 °C. This intermediate product was analyzed by ESI−MS (calcd 25259.0 Da, found 25258.0 Da, Figure 3c), and directly treated with $MeONH_2$·HCl at pH 3−4 (400 mM final concentration, 16 h) for the thiazolidine opening reaction. The protein was subsequently purified by FPLC (HiTrap desalting columns, AcOK pH 4.5 eluting buffer). ESI−MS (calcd 25247.0 Da, found 25246.0 Da, Figure 3d). The overall yield of modified antibody recovered was 71%.

General Procedure for Thiazolidine formation. The protein solution in AcOK (pH 4.5, 100 mM) was concentrated with centrifugal filter units from Millipore (Amicon, Ultra centrifugal filter, 0.5 mL; Ultracel, 3 kDa membrane; Millipore; UFC500324) until an antibody concentration of 20 μM was reached. The concentrated proteins were first reduced with 1 mM DTT or 1 mM TCEP·HCl (16 h), and then incubated with cemadotin aldehyde (Cem-CHO, 40 mM stock in EtOH, 1−4 mM final concentration, 10% max EtOH concentration, 40−200 equiv). The reaction mixture was incubated at 4 °C for 2.5−4 days, monitoring conversion by LC−MS, and then purified by FPLC (Pharmacia Akta-FPLC system) with two HiTrap desalting columns in series (GE Healthcare) of 5 mL volume equilibrated with PBS as running buffer at a flow rate of 1 mL/min. The fractions containing the antibody were collected, concentrated (absorbance at 280 nm), and used for release experiments or in vitro cytotoxicity assay. ESI−MS: Cem-thz-Db(F8) (calcd 25541.0 Da, found 25542.0 Da, Figure 5b). Cem-thz-Gly-Db(F8) (calcd 25598.0 Da, found 25598 Da, Figure 5c); Db(F8)-Cys-M-thz-Cem (calcd 25898.0 Da, found 25900.0 Da, Figure 6b).

Drug Release Experiments. ADCs were tested for stability and drug release. Aliquots (110 μL, 5−10 μM concentration in PBS) were incubated at three different temperatures (i.e., 37, 4, −80 °C) and treated at given time points as follows. The aliquots (thawed if necessary) were shortly centrifuged (4 °C, 1 min, 14 000 rpm), the supernatant was transferred to a 1.5 mL Eppendorf tube, then 5.5 μL of DMSO was added (to reduce unspecific interactions with resin particles). The solution was incubated with protein A resin (50 μL from 50% slurry, prewashed twice with 2× 500 μL of 5% DMSO in PBS). The mixture was incubated at 4 °C for 20 min in a thermomixer; then, the resin was removed by gentle centrifugation (1 min, 600*g*). For each time point in the study, 100 μL of supernatant was withdrawn, shock frozen, and stored at −20 °C until further analysis. The concentration of released Cem-CHO was determined by tandem LC−MS/MS analysis, using a Multiple Reaction Monitoring (MRM) technique. Standard solutions of the synthetic Cem-CHO (25−0.05 μM) were used to obtain a calibration curve and samples were treated exactly as for the drug aliquots released from the corresponding ADC. Prior to analysis, samples were acidified (final [HCl] = 100 mM) and analyzed on a Micromass Quattro micro API using an XTerra MS C_{18} column, 3.5 μM, 1.0 × 50 mm (Waters; 186000386) and run on a linear gradient from 0 to 90% acetonitrile (B) in 0.1% formic acid (A) in 11 min. For MRM analysis, daughter ions with *m*/*z* 85.98, 100.3, 227.43, and 340.5 of the parent ion 669.43 from Cem-CHO were monitored, comparing peak areas to those of the Cem-CHO calibration series.

In Vitro Cytotoxicity Assays. HEK 293, HL60 (CCL-240, ATCC), and WI38VA-13 cells (CCL-75.1, ATCC) were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco-Invitrogen) medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco-Invitrogen). F9 murine teratocarcinoma cells (CRL-1720, ATCC) were grown in RPMI (Gibco-Invitrogen) media supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco-Invitrogen) in culture dishes coated with 0.1% gelatin. All cell lines were maintained at 37 °C in 5% CO_2 atmosphere. For the in vitro cytotoxicity assay, 10 000−15 000 cells of each cell line were seeded in 100 μL into a multiwell plate and a defined volume of the appropriate dilution of cemadotin or cemadotin products (e.g., Cem-CHO, and Cemadotin ADCs) was added. After incubation at 37 °C in 5% CO_2 for 72 h, cell viability was determined with the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega). Twenty microliters of this solution was added to each well, and after 2 h of incubation at 37 °C in 5% CO_2, absorption was measured at 490 nm. The percentage of cell growth inhibition was calculated as a ratio of the counts between treated cells over untreated cells.

RESULTS

Preparation of 1,2-Aminothiol Containing Antibodies. To facilitate analytical studies in the absence of protein glycosylation, we used recombinant antibodies in diabody format,[18] produced in CHO cells using transient gene expression (TGE).[16] For chemical transformations, we used the clinical-stage human monoclonal antibody F8, which is specific to the alternatively spliced EDA domain of fibronectin, a marker of tumor angiogenesis.[19,20]

One strategy for the introduction of a 1,2-aminothiol moiety in the antibody molecule consisted in the introduction of a cysteine residue immediately after the cleavage site of the leader peptide required for antibody secretion (Figure 2). This strategy led to the production of F8 variants (termed Cys-Db(F8)), which contained an N-terminal cysteine residue separated from the main antibody by a variable number of glycine residues (*n* = 0, 1, 4). The best results in terms of protein homogeneity and expression yields were obtained with the shortest N-terminal sequences (Cys- or Cys-Gly-, Figure 2). Closely related protein species were observed by MS analysis after antibody purification (Figure 2d,g), which were converted into a single molecular species of the predicted molecular weight upon treatment with DTT (10 mM) as reducing agent, followed by treatment with $MeONH_2$·HCl (400 mM, Figure 2e,h).[21]

A second strategy for the introduction of the 1,2-aminothiol moiety into the F8 antibody in diabody format featured the chemical modification of an engineered C-terminal cysteine residue (Db(F8)-Cys)[17] by means of a newly developed maleimide/thiazolidine (M-thz) heterobifunctional linker (Figure 3). After a mild reduction of the covalent interchain disulfide bond[17,22,23] of Db(F8)-Cys with TCEP·HCl (1 mM), the resulting thiol was reacted with the M-thz linker, yielding a modified antibody carrying a thiazolidine moiety (Db(F8)-Cys-M-thz; Figure 3a). The reaction was selective and quantitative, as shown by SDS−PAGE and ESI−MS analysis (Figure 3b,c). The thiazolidine group was then deprotected with a molar excess of $MeONH_2$·HCl (400 mM final concentration, pH 3−4) to afford the desired 1,2-aminothiol (Db(F8)-Cys-M-Cys, Figure 3d), suitable for subsequent modification with aldehydes, in good yields.

Cemadotin Aldehyde (Cem-CHO). To generate a potent cytotoxic drug containing an aldehyde moiety for thiazolidine formation, we synthesized a dolastatin-15 analogue (Figure 4a, R═CHO; see SI for synthetic details), based on a conservative modification of the corresponding cytotoxic drug cemadotin (Figure 4a, R═H).[24] Dolastatins are a class of small peptides, isolated from the shell-less mollusk *Dolabella auricularia*, that bind to tubulin subunits, inhibit microtubule assembly, and depolymerize existing microtubules, thus, blocking cell cycle progression during mitosis at the G_2−M phase. The depsipeptide dolastatin-15 is one of the most studied members of this family. However, this drug showed no significant benefits for patients with advanced solid malignancies, possibly due to poor cellular uptake and inadequate pharmacokinetics.[25] Cemadotin is a highly toxic, water-soluble analogue of dolastatin-15 with simplified peptidic structure and a C-terminal benzylamine moiety in place of the original pyrrolidone. This drug has been studied as anticancer agent in numerous clinical trials, but its narrow therapeutic window has hampered advanced clinical developments.[25] The

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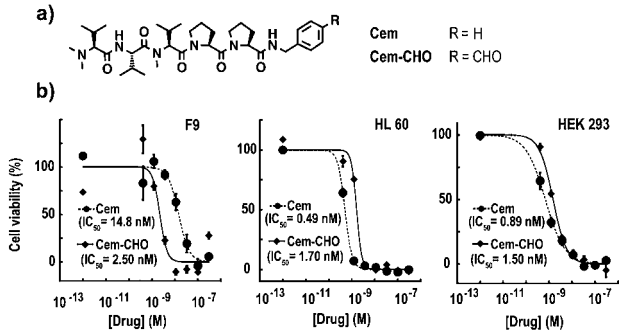

Figure 4. (a) Chemical structure of cemadotin (R═H, Cem) and the corresponding aldehyde derivative (R═CHO, Cem-CHO). (b) In vitro cytotoxicity test. Cytotoxicity of Cem and Cem-CHO was measured by adding the compounds at various concentrations to F9, HL60, and HEK 293 cell lines. Cells were incubated for 72 h at 37 °C in 5% CO_2 atmosphere. Cell viability was then monitored with the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega); values were read after 2 h. The figure shows that the cell killing profiles for Cem and Cem-CHO are comparable.

cemadotin aldehyde analogue (Figure 4a; hereafter named Cem-CHO) retained the cell killing activity of the parental cemadotin compound toward a different panel of cell lines, with significant potency in the low nanomolar range (Figure 4b).

Thiazolidine Linkage Formation. Our general conjugation strategy (Figures 5a and 6a) relied on thiazolidine linkage

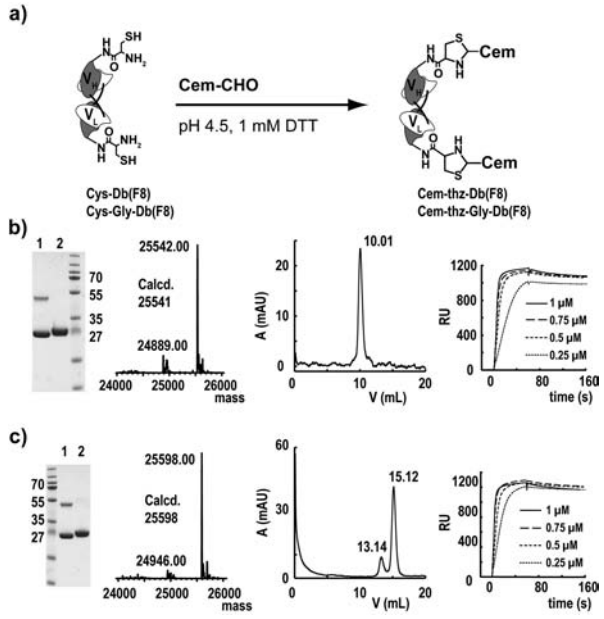

Figure 5. (a) Schematic representation of Cys-Db(F8) and Cys-Gly-Db(F8) diabodies reacting with Cem-CHO to form chemically defined thiazolidine conjugates. (b and c) Cys-Db(F8) and Cys-Gly-Db(F8) coupling products with Cem-CHO, respectively, analyzed by SDS–PAGE (lanes 1 and 2, nonreducing and reducing conditions), ESI–MS, size-exclusion chromatography (Superdex 75 HR 10/300 GL column and Superdex 200 HR 10/300 column), and Biacore technology on microsensor chip coated with EDA-containing 11A12 fragment.[17]

formation between the 1,2-aminothiol containing diabodies and Cem-CHO in AcOK buffer at pH 4.5 under mild reducing conditions (1 mM DTT or TCEP·HCl). After 2.5–4 days, we observed conversions to the desired antibody-drug conjugates with yields in excess of 90% as confirmed by ESI–

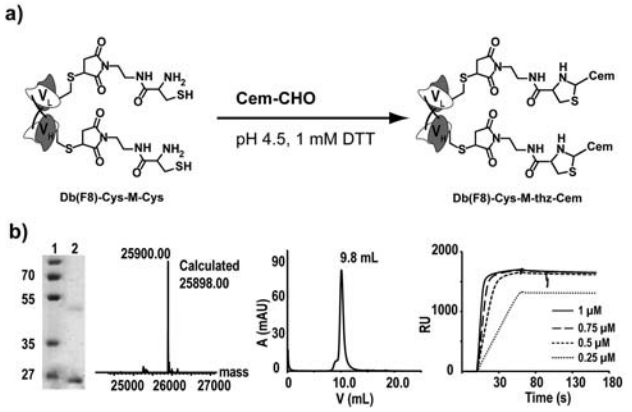

Figure 6. (a) Schematic representation of Db(F8)-Cys-M-Cys diabody reacting with Cem-CHO to form chemically defined thiazolidine conjugates. (b) Db(F8)-Cys-M-thz-Cem coupling product, analyzed by SDS–PAGE (lane 2, nonreducing conditions), ESI–MS, size-exclusion chromatography (Superdex 75 HR 10/300 GL column), and Biacore on microsensor chip coated with EDA-containing 11A12 fragment.[17]

MS (Figures 5b,c and 6b). The resulting chemically defined conjugates, Cem-thz-Db(F8), Cem-thz-Gly-Db(F8), and Db(F8)-Cys-M-thz-Cem, displayed excellent purity, stability, and antigen binding properties, as confirmed by SDS–PAGE, ESI–MS, size-exclusion chromatography, and Biacore analysis (Figures 5b,c and 6b).

Cem-CHO Release Experiments. Having established reliable methods for the site-specific modification of antibodies with a potent analogue of cemadotin, we evaluated the kinetics of drug release and the resulting cytotoxicity of the cleavable ADCs synthesized from N-terminal cysteine modified antibodies (Cem-thz-Db(F8) and Cem-thz-Gly-Db(F8)). Cem-CHO release from both chemically modified antibodies was monitored at 37 °C in PBS by tandem LC–MS/MS spectrometry (Figure 7a). The half-life for the Cem-thz-Gly-Db(F8) conjugate (45 h) yielded ~65% of free drug after 75 h (pure Cem-CHO was used as positive control for MS quantification of the release process). Both ADCs were stable when stored at low temperature, as confirmed by release kinetics measured at 4 °C and −80 °C (SI).

We evaluated the Cem-CHO release process in cytotoxicity assays with WI38VA-13 and HL60 cell lines. These cells do not enable an antibody-mediated internalization of the drug and were chosen to test in vitro the cell killing ability of Cem-CHO released from the corresponding thiazolidine-based ADC. Unmodified Cem-CHO was used as positive control in the experiment, while antibody conjugates with cemadotin linked via a noncleavable amide bond, synthesized by native chemical ligation (SI), were used as negative controls. As expected, Cem-CHO released from the thiazolidine-based conjugates mediated a cell killing activity almost comparable to the one of the same drug administered at once to the cell culture as pure chemical reagent. By contrast, as expected, ADCs with stable amide bonds exhibited a substantially lower cytotoxicity (Figure 7b).

■ DISCUSSION

Chemoselective reactions are of fundamental importance for the generation of post-translationally modified proteins and for the production of antibody–drugs conjugates. The thiazolidine formation reaction has been known to chemists for decades but had previously never been used for the site-selective preparation of antibody–drug conjugates, capable of a slow release of a potent

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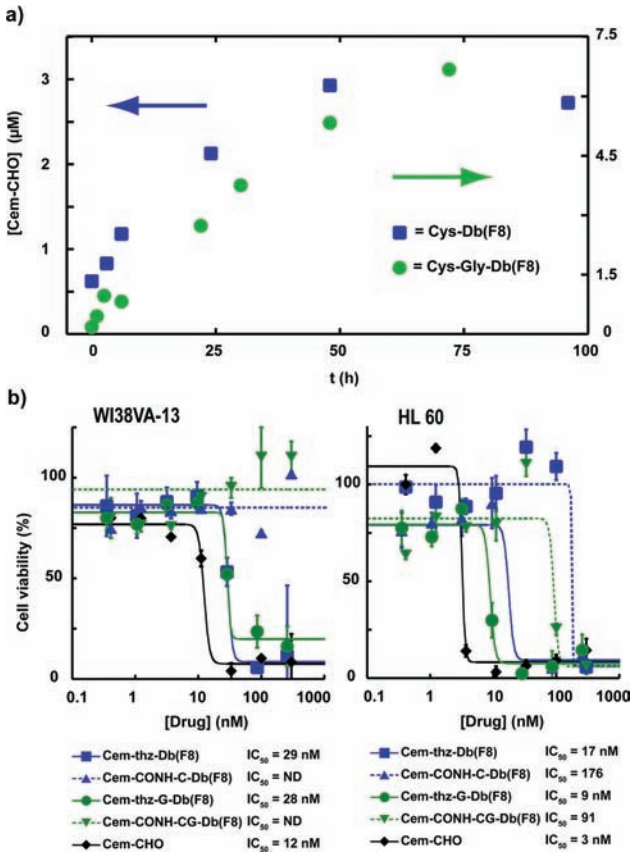

Figure 7. Drug release experiments from thiazolidine-based ADCs. (a) LC−MS/MS quantification of Cem-CHO released from Cem-thz-Db(F8) (■; left *y* axis; starting ADC concentration of 5.0 μM) or Cem-thz-Gly-Db(F8) (●; right *y* axis; starting ADC concentration of 10.0 μM). Samples were incubated at 37 °C in PBS, the residual ADC and the free antibody were removed by affinity capture on protein-A resin, and the released Cem-CHO was analyzed and quantitated at different time points using Multiple Reaction Monitoring (MRM) technique. Values measured were compared with a calibration curve generated from a standard dilution series of the free Cem-CHO drug. (b) In vitro cytotoxicity assay of Cys-Db(F8) (blue) and Cys-Gly-Db(F8) (green) conjugates with Cem-CHO, performed on WI38VA-13 and HL60 cells. The cell killing activity mediated by Cem-CHO released from thiazolidine-based conjugates (solid lines) was compared to the one observed with amide bond-based conjugates (dashed lines) and the free Cem-CHO drug (black). Cytotoxicity of Cem-CHO and of the conjugates was measured by adding the compounds at various concentrations to WI38VA-13 and HL60 cell lines and determining their vitality, as described in Methods. Amino acids are indicated using single letter codes: C = Cys; G = Gly; CONH indicates the amide bond.

cytotoxic agent. Oximes, hydrazones, and thiazolidines are stable aldehyde derivatives, with the latter being the most stable form in a subclass of glyoxal derivatives.[8] The stability of hydrazones formed from aldehydes with electron-withdrawing substituents is maximal.[26] In this context, the benzaldehyde moiety present in Cem-CHO displays intermediate reactivity values, thus, yielding derivatives sufficiently stable for synthesis and storage, while permitting a hydrolytic drug release under physiological conditions at 37 °C. The half-life of Cem-thz-Gly-Db(F8) (45 h) is long compared to the circulatory half-life of Db(F8) in vivo following intravenous administration in tumor-bearing mice ($T_{1/2} \sim 30$ min), but is short compared to the residence time of the antibody at the tumor site (several days).[20,27] Thus, the thiazolidine linker appears to be ideally suited for the site-specific coupling of cytotoxic aldehydes to tumor-targeting antibodies (such as F8) in the diabody format. Diabodies display superior biodistribution properties compared to other antibody formats, which have already been validated in quantitative biodistribution experiments with radiolabeled antibody preparations.[28−30] The introduction of an N-terminal cysteine residue, following leader peptide cleavage, is a general strategy which is applicable to other antibody formats (Figure 1) and is compatible with standard antibody manufacturing procedures. Alternatively, 1,2-aminothiols can be introduced by chemical transformation of cysteine-modified recombinant antibodies using the newly developed M-thz bifunctional linker (Figure 3), but the biosynthetic approach described in Figure 2 is more attractive, as it requires fewer chemical modification steps.

To our knowledge, this is the first example in which a thiazolidine linkage was used as cleavable linker for the targeted delivery and slow release of cytotoxic drugs.

The antibody−drug conjugates, prepared using antibodies carrying an engineered Cys residue at the N-terminus, were assembled in a single site-specific, chemoselective step. Upon thiazolidine cleavage, the native antibody and the free drug are restored, leaving no "scar" from the original linkage. This chemical feature facilitates the pharmacokinetic analysis of the drug release process and limits immunogenic reactions, which may be associated with the presence of residual linker moieties on the antibody.[31−33]

We have recently generated and characterized antibody conjugates based on thiol-containing drugs and mixed disulfides as a second example of cleavable traceless linkers.[34] Pharmaceutical agents containing an aldehyde moiety have been approved in the categories of antibiotics,[35] antibacterials,[35] and antiallergics.[36] Additional aldehyde drugs are under active development. One of them, Gossypol (AT101), is a potent Bcl-2 antagonist which is currently being investigated in phase II studies against various tumor types. AT101 possesses two aldehyde groups that are responsible for interactions with different biomolecular targets.[37] Masking aldehydes by conjugation to antibodies is an attractive pharmaceutical strategy, as it protects the aldehyde function from reaction with bioavailable nucleophiles and from oxidation.

The use of aldehyde drugs and of thiazolidine linkers may be particularly suited for the selective killing of cancer stem cells by antibody−drug conjugates, considering the fact that aldehyde dehydrogenase (ALDH) activity represents one of the most prominent characteristics of "cell stemness".[38,39] ALDH-mediated chemical transformation of membrane-permeable aldehyde derivatives of fluorophores into their corresponding carboxylic acids (which remain trapped in the cell) has long been used to selectively label stem cells and for fluorescence-activated cell sorting procedures.[40,41] It would therefore be feasible to use thiazolidine linkers for the coupling of aldehyde drugs with internalizing human antibodies capable of selective recognition of cancer stem cells. Those tumor cells are characterized by an unlimited regeneration potential and are suspected to be responsible for resistance to chemotherapeutic treatment and relapse.[42−44] The intracellular conversion of aldehyde drugs into the corresponding carboxylic acids is likely to be associated with a potent cytotoxic effect, if the acids remain bioactive and if their exit from the cell is hindered by their negative charge at neutral pH.

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■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, characterization data, 1,2-aminothiol incorporation with cross-linkers, native chemical ligation conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Giulio Casi is employed at Philochem AG, Dario Neri is a co-founder and shareholder of Philogen SpA.

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