VIP Cancer Therapy

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A Traceless Vascular-Targeting Antibody–Drug Conjugate for Cancer Therapy**

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Monoclonal antibodies have demonstrated considerable utility in the clinical treatment of cancer,[1] but unmodified immunoglobulins are rarely curative, especially when used as single agents. Thus, there is considerable interest in arming antibodies with bioactive payloads (e.g., drugs, radionuclides, cytokines), to improve their potency and selectivity, thus increasing activity at the tumor site while sparing normal tissues.[2,3]

Significant progress has been made in the past few years in the area of antibody–drug conjugates (ADCs) for the selective delivery of cytotoxic drugs to tumors.[4,5] As a result of these investigations, new agents with pronounced clinical activities have been developed, including SGN-35 (an ADC directed against CD30-positive hematological malignancies)[6] and trastuzumab-DM1 (which has shown activity in metastatic breast cancer).[7] As conventional drug conjugation strategies yield heterogeneous ADC preparations, intense efforts are being devoted to the development of methods for site-selective modification of therapeutic antibodies, thus leading to products with improved performance and batch-to-batch reproducibility.[8] Furthermore, comparative evaluations of intact immunoglobulins in IgG format and other recombinant antibody formats for ADC development have been conducted.[9,10]

It is generally assumed that ADCs may need to be internalized by the tumor cells for the active release of cytotoxic drugs.[4,5] Once ADCs are internalized and the drug is released in the intracellular compartments, a cross-fire effect (corresponding to the migration to neighboring cells) may occur, as has been reported for the treatment of tumors consisting of a mixture of antigen-positive and antigen-negative cells.[11] However, monoclonal antibodies specific to tumor cell antigens often exhibit limited diffusion into the solid tumor mass by several mechanisms, including slow extravasation and antibody trapping by perivascular tumor cells (the so-called antigen barrier).[12] In view of the fact that the formation of new blood vessels (angiogenesis) is a rare process in a healthy adult but a characteristic feature of virtually all types of aggressive cancers, it would be reasonable to develop vascular-targeting ADCs.[3,13,14] Unlike the use of cell-specific markers, vascular targeting offers comprehensive tumor coverage, as the majority of cancers express splice isoforms of tenascin-C and of oncofetal fibronectin.[15] In addition, vascular targeting helps address the issue of heterogeneity of antigen expression within the tumor mass (i.e., tumor cells which are positive or negative for the antigen). Despite the remarkable potency of cytotoxic compounds targeting the tumor vasculature and the strong dependence of growing neoplastic masses on florid angiogenesis, only limited efforts were directed in the past towards the investigation of ADCs that target tumor vascular antigens.[16] We have recently shown that the antibody-based delivery of photosensitizers to tumor blood vessels and irradiation may induce complete and long-lasting cancer eradication, in a process that also involves the action of natural killer cells.[17] Thus, there appears to be a strong rationale for the targeted delivery of cytotoxic agents to the tumor neovasculature for cancer therapy.

Given that antibodies are large molecules compared to cytotoxic agents, potent drugs need to be used to generate ADCs that can be administered at reasonably low doses and that are compatible with industrial development activities at acceptable cost of goods.[4] Herein, we aimed at generating a novel class of chemically defined vascular-targeting ADCs that release cytotoxic drugs with a mechanism that does not require antibody internalization. We reasoned that ADCs based on linkerless antibody modification with a potent thiol-containing drug would allow the formation of homogeneous products by the formation of a mixed disulfide. These agents could release the cytotoxic payload in the extracellular space, when tumor cell death is initiated and releases high concentrations of reducing agents (e.g., cysteine, glutathione) to the surrounding environment. Provided that a sufficiently large amount of ADC can be delivered to the subendothelial extracellular matrix, the drug release process would be amplified as tumor cell death progresses.

Dolastatins are a group of small peptides isolated from the Indian ocean hare *Dolabella auricularia*[18,19] that bind to tubulin subunits and inhibit new microtubule assembly and depolymerize existing microtubules, thus blocking cell cycle

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progression during mitosis.[20] Initial clinical investigations of dolastatin 10, a linear pentapeptide, and dolastatin 15, a seven unit depsipeptide, showed no significant benefit in patients with advanced solid malignancies, possibly as a result of poor cellular uptake and inadequate pharmacokinetics.[20] Analogues of dolastatins were thus synthesized, in an attempt to improve in vivo performance. For example, auristatin PE, cemadotin, and tasidotin were synthesized by variation of the amide moiety at the C terminus of the molecule and have been studied in clinical trials in patients with cancer.[20] Cemadotin (Figure 1a, R = H), a water-soluble analogue of dolastatin 15 with a terminal benzylamine moiety instead of

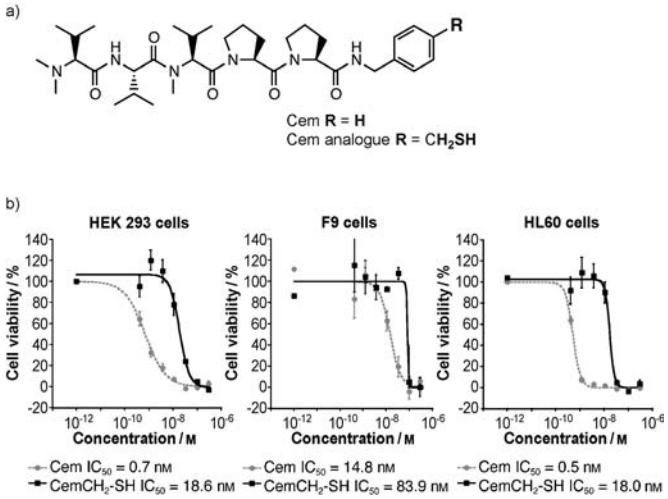

Figure 1. CemCH$_2$-SH, a potent cytotoxic analogue of cemadotin. a) Chemical structure of cemadotin (Cem; R = H) and the synthetic analogue CemCH$_2$-SH (R = CH$_2$SH) used in this study. The analogue CemCH$_2$-SH was synthesized using peptide coupling chemistry starting from *S*-4-(aminomethyl)benzyl ethanethioate and the pentapeptide P5, which was assembled by step-wise solid-phase peptide synthesis. Hydrolysis of the thioester gave the desired thiol drug. See the Supporting Information for detailed synthetic procedures and characterization. b) Bioactivity assay of Cem and its analogue CemCH$_2$-SH. The biological activity of the two drugs was determined by their ability to inhibit proliferation of cells.

the original pyrrolidone, is one of the most potent cytotoxic agents described so far.[21] This potent cytotoxic activity together with its simple chemical structure (when compared to other known anticancer drugs) makes cemadotin an ideal candidate for use in our vascular-targeting ADC strategy. We reasoned that cemadotin could be synthetically manipulated, by introduction of a thiol reactive tag for disulfide site-selective conjugation to an antibody,[22,23] without compromising its activity. Thus, a thiol-containing cemadotin analogue (CemCH$_2$-SH, R = CH$_2$SH; Figure 1a) was designed and synthesized using peptide coupling chemistry and starting from *S*-4-(aminomethyl)benzyl ethanethioate and the pentapeptide P5, which was assembled by step-wise solid-phase peptide synthesis. A final hydrolysis of the thioester gave the desired thiol drug (see the Supporting Information for synthetic details). The in vitro potency of CemCH$_2$-SH was evaluated in different cell lines and demonstrated to retain cytotoxic activity in the low nanomolar concentration range comparable to cemadotin (Figure 1b).

As a tumor-targeting moiety we used the human antibody F8, which is specific to the EDA domain of fibronectin, a marker of tumor angiogenesis. The antibody was used in a small immune protein (SIP) format,[24,25] which displays superior tumor-targeting properties compared to IgGs and smaller antibody fragments, as evidenced by quantitative biodistribution studies[26] and by nuclear medicine investigations.[27] The antibody SIP(F8) can be produced in high yields in mammalian cells (see the Supporting Information) and contains a cysteine residue at the C-terminal position of each εCH4 domain, which can be chemically modified without loss of immunoreactivity or tumor-targeting performance.[28]

Based on its relative ease of access and reversibility under mild reducing conditions, we decided to pursue a disulfide strategy for site-selective antibody modification.[22,23] The C-terminal cysteine residues involved in the interchain covalent disulfide bond, may be reduced under mild conditions for further chemical manipulation without affecting the intra-domain disulfide bridges and antibody binding, because the C-terminal cysteine residues are remote from the binding site. In addition, the resulting noncovalent homodimer remains kinetically stable after chemical modification (see below). Our general conjugation strategy (Figure 2a) involved mild reduction of the covalent disulfide bond using tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) followed by activation of the resulting cysteine residues with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Ellman's reagent). Subsequent incubation with as little as 10 equivalents of CemCH$_2$-SH for 10 minutes at room temperature in phosphate-buffered saline (PBS) pH 7.4 with purified SIP(F8)–Ellman's yielded a homogeneous mixed disulfide, which we denote SIP(F8)-SS-CH$_2$Cem. The low pK_a of the aromatic thiol accelerated formation of the mixed disulfide at neutral pH and prevented competing formation of a symmetric disulfide. This conjugation method proceeded with complete conversion (> 95%) within 10 minutes (see the Supporting Information), thus enabling the preparation of ADCs with excellent overall yields. The resulting chemically defined, linkerless SIP(F8)-SS-CH$_2$Cem conjugate displayed excellent purity and antigen-binding properties, as confirmed by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Figure 2b), ESI-MS analysis (Figure 2c), size-exclusion chromatography (Figure 2d), and Biacore studies (Figure 2e). The conjugate was demonstrated to be stable under storage conditions (−80 °C and 4 °C) and could be detected in mouse plasma even after 72 hours incubation at 37 °C as evidenced by ESI-MS analysis (see the Supporting Information). In addition, SIP(F8)-SS-CH$_2$Cem readily reacted in the presence of as little as 0.3 mM reduced glutathione (see the Supporting Information). This observation is of particular importance since we expect the presence of small amounts of physiological reducing agents (e.g., cysteine, glutathione) to trigger disulfide bond reduction and the consequent release of the cytotoxic payload from the antibody.

Having established a robust method for the site-selective modification of SIP(F8) with a potent cytotoxic analogue of

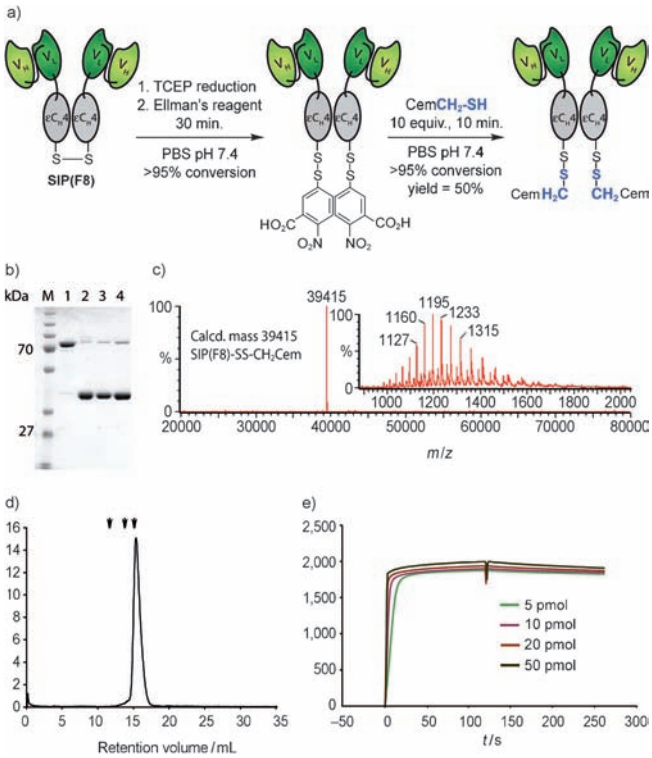

Figure 2. Construction of a traceless, chemically defined tumor-vasculature-targeting antibody–drug conjugate (ADC). a) Schematic illustration for the generation of a chemically defined tumor-vasculature-targeting ADC. The F8 antibody is specific to the EDA domain of human and murine fibronectin. b) SDS-PAGE analysis of site-selective conjugation of $CemCH_2$-SH to SIP(F8): M = molecular marker; 1) unreduced SIP(F8); 2) SIP(F8) reduced with TCEP; 3) purified SIP(F8)–Ellman's; 4) purified SIP(F8)-SS-CH_2Cem. c) ESI-MS spectrum of purified SIP(F8)-SS-CH_2Cem. Ion count (main spectrum) = 6.07×10^5, ion count (inset) = 6.10×10^5. d) Gel-filtration analysis of purified SIP(F8)-SS-CH_2Cem. The peak eluting at a retention volume of 15.3 mL corresponds to the noncovalent homodimeric form of SIP(F8)-SS-CH_2Cem. Arrows indicate standard proteins (11 mL: Ferritin 440 kDa; 14.1 mL: BSA 67 kDa; 15.4 mL: β-lactoglobulin 35 kDa). e) Biacore analysis of purified SIP(F8)-SS-CH_2Cem towards recombinant 11A12 fibronectin.

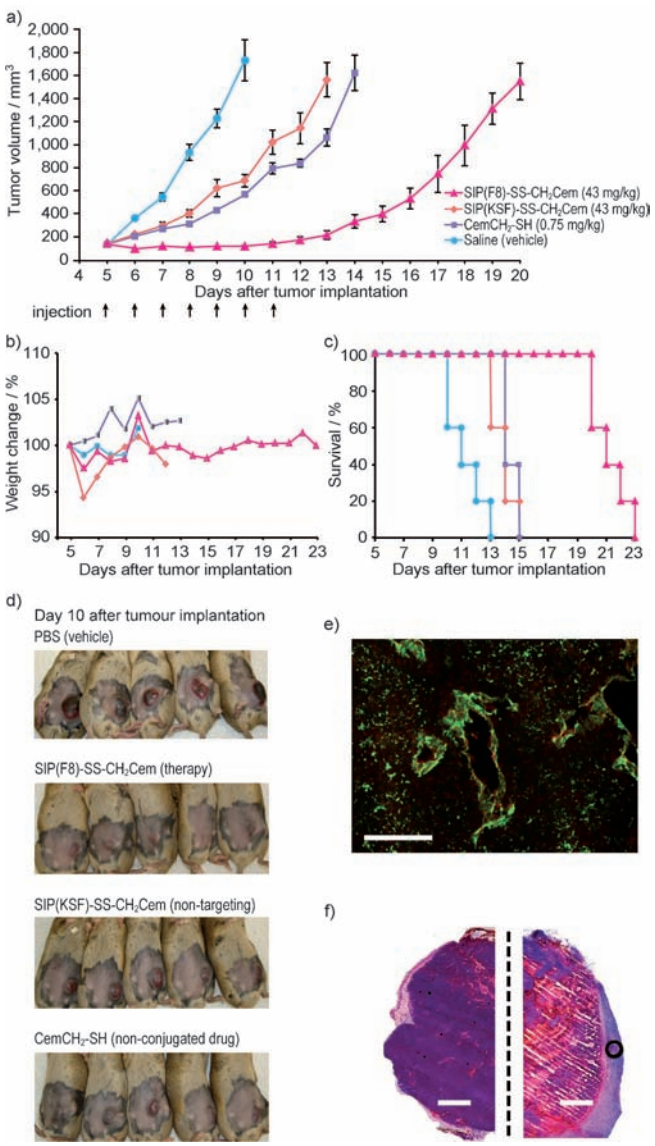

Figure 3. Therapeutic efficacy of SIP(F8)-SS-CH_2Cem in a syngeneic murine tumor model. a) Immunocompetent 14-week-old female 129SvEv mice bearing subcutaneous F9 teratocarcinomas were treated intravenously with 960 μg of SIP(F8)-SS-CH_2Cem (targeting EDA), 960 μg of SIP(KSF)-SS-CH_2Cem (specific to an irrelevant antigen), 18 μg of unconjugated $CemCH_2$-SH or saline (5 mice per group). Treatment was performed daily for a period of 7 days (arrows). Therapy was initiated when tumors reached a size of 125 mm^3. Data represents mean tumor volumes (± standard error). Tumor growth curves were stopped when tumors reached a size of 1800 mm^3. b) Body weight variations of the mice during and after therapy. No detectable weight loss was observed. c) Survival curves of treatment and control groups; substantial prolongation for mice which received SIP(F8)-SS-CH_2Cem. d) Photos of mice bearing subcutaneous F9 teratocarcinomas on day 10 after tumor implantation, after having received five daily treatments. e) Fluorescence microscopy analysis of F9 tumor sections, co-stained for EDA and endothelial cells with SIP(F8) (green) and rat anti-mouse CD31 antibody (red), respectively. SIP(KSF) was used as negative control (see the Supporting Information). Scale bar, 100 μm. f) Effect of a single injection of SIP(F8)-SS-CH_2Cem on tumor histology. Sections of F9 tumors were excised 24 h after a single intravenous injection of 960 μg SIP(F8)-SS-CH_2Cem, stained with haematoxylin/eosin and analyzed by light microscope. Left (saline group), Right (treated tumor). Scale bar, 2 mm.

cemadotin, we evaluated the anticancer activity of this vascular tumor-targeting ADC in immunocompetent Sv129Ev mice, bearing subcutaneously grafted F9 tumors. As negative controls, groups of mice ($n = 5$) received injections of saline, unconjugated $CemCH_2$-SH, or SIP(KSF)-SS-CH_2Cem, an ADC which recognizes an antigen of irrelevant specificity in the mouse (hen egg lysozyme; see the Supporting Information).[29] At 8 $mg\,kg^{-1}$ doses of SIP(F8)-SS-CH_2Cem, around 50% tumor growth retardation was observed (see the Supporting Information, Figure S1a and b). The use of the antibody in SIP format was shown to be preferable when compared to the recombinant diabody(Db)-Cys format (see the Supporting Information, Figure S2 and S1c and d). Encouraged by these results, a new therapy experiment was performed using 43 $mg\,kg^{-1}$ doses of the conjugate (corresponding to 18 μg of $CemCH_2$-SH per mouse). Under these conditions, a very potent tumor growth retardation was observed for SIP(F8)-SS-CH_2Cem,

which was significantly superior ($P < 0.05$) compared to all three control groups (Figure 3a and d).[30] The treatments, which were very well tolerated, with no detectable weight loss (Figure 3b), led to a substantial prolongation of survival for mice that received SIP(F8)-SS-CH_2Cem (Figure 3c). As this tumor-targeting ADC selectively recognizes and localizes to neovascular structures in vivo (Figure 3e), we studied at the microscopic level the effect of a single injection of SIP(F8)-SS-CH_2Cem. Similar to what was previously reported for vascular disrupting agents[31] and for antibody-photosensitizer conjugates,[17] a rapid conversion of the internal core of the tumor mass into a large necrotic area was observed (Figure 3f).

In conclusion, a novel approach for the delivery of cytotoxic drugs using ADCs has been developed. Current ADCs target tumor cell surface markers and rely on the ADC being internalized in the cells for drug delivery. Instead, our approach relies on targeting the tumor neovasculature offering comprehensive tumoral coverage. The site-selective chemistry used gives defined products conjugated directly at cysteine by a disulfide bridge. Unlike current strategies for ADC preparation, our strategy does not require the use of elaborate linkers that complicate chemical synthesis and may be immunogenic. Additionally, our approach leads to a traceless ADC from which only two species will result upon cleavage of a disulfide bridge, the native antibody and free drug.

To our knowledge, this is the first experimental demonstration that a noninternalizing vascular-targeting ADC can be used to mediate a strong antitumor activity in vivo. The traceless ADC presented here allows the progressive amplification of drug release, as tumor cell lysis mediates glutathione and cysteine release to the surrounding tissue. It is likely that ADCs that target the tumor vasculature instead of cell surface markers may offer additional opportunities for cancer therapy, as angiogenesis is a common feature of virtually all types of aggressive cancers.

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