A chemically-defined trifunctional antibody-cytokine-drug conjugate with potent anti-tumor activity

Running Title: An antibody-cytokine-drug conjugate with anti-tumor activity

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Abstract

The combination of immunostimulatory agents with cytotoxic drugs is emerging as a promising approach for potentially curative tumor therapy, but advances in this field are hindered by the requirement of testing individual combination partners as single agents in dedicated clinical studies, often with suboptimal efficacy. Here we describe for the first time a novel multi-payload class of targeted drugs, the immunocytokine-drug conjugates (IDCs), which combine a tumor-homing antibody, a cytotoxic drug and a pro-inflammatory cytokine in the same molecular entity. In particular, the IL2 cytokine and the disulfide-linked maytansinoid DM1 microtubular inhibitor could be coupled to the F8 antibody, directed against the alternatively-spliced EDA domain of fibronectin, in a site-specific manner, yielding a chemically defined product with selective tumor homing performance and potent anticancer activity in vivo, as tested in two different immunocompetent mouse models.
Introduction

Modern pharmacotherapy of cancer increasingly makes use of drug combinations, which may display a synergistic therapeutic activity in vivo, acting through complementary mechanisms of action (1-5). In practice, however, the combination of pharmaceutical agents is difficult to implement in the clinic, since the individual drugs typically need to be extensively studied as single agents, before they can be considered for combination trials. This procedure is expensive, time-consuming and may deny patients a timely access to pharmacologically active treatments.

Cytotoxic agents remain the backbone of cancer pharmacotherapy. In many cases, cytotoxic drugs represent ideal combination partners for immunostimulatory agents (e.g., checkpoint inhibitory antibodies, cytokines), since the two classes of compounds exhibit different and mutually compatible toxicity profiles, while exploiting complementary mechanisms of action. In preclinical studies, the therapeutic activity of IL2-based immunocytokines was strongly potentiated by the combination with the mitotic inhibitor paclitaxel (2, 6), doxorubicin (6), temozolomide (7) and with sunitinib (8). In addition, tumor-targeting immunocytokines are being studied in clinical trials in combination with dacarbazine (9), gemcitabine (9-11), melphalan (12), fenretinide (13), doxorubicin (6) and paclitaxel (14, 15).

Emerging experimental evidence indicates that cytotoxic drugs may act on the tumor endothelium and favor the extravasation of therapeutic agents, while also promoting the expression of stress proteins on cancer cells, which can be recognized by leukocytes in vivo. For example, increased vascular permeability (2), reduction of interstitial fluid pressure (16, 17), increased infiltration and local activation of leukocytes (2, 18) have been proposed as key determinants for the anti-tumor activity of paclitaxel, one of the most broadly used anti-cancer drugs with microtubular inhibitory activity.

The therapeutic index of both cytotoxic drugs and cytokines can be strongly enhanced when these potent payloads are coupled to tumor-homing antibodies, serving as selective pharmacodelivery agents (10). Antibody-drug conjugates (ADCs) are armed versions of monoclonal antibodies, which may deliver a highly-potent cytotoxic agent at the tumor site, thus helping spare normal tissues (19, 20). Similarly, a large number of pro-inflammatory cytokines has been genetically fused to tumor-targeting recombinant antibodies, yielding fusion proteins
(termed “immunocytokines”) which display a potent therapeutic activity in mouse models of the disease and encouraging results in cancer patients (6, 7, 9, 11, 14, 15, 21-27).

For many applications, antibodies specific to splice isoforms of extracellular matrix components may represent ideal vehicles for the delivery of both cytotoxic drugs and cytokines (28-32). For example, the alternatively-spliced EDA domain of fibronectin is virtually undetectable in normal adult tissues (exception made for placenta, for the endometrium in the proliferative phase and for some vessels in the ovaries (33)), but is strongly expressed around neo-vascular structures and in the interstitium of most aggressive solid tumors (34), lymphomas (35) and acute leukemias (36). In particular, the F8 monoclonal antibody recognizes the human and murine EDA domain of fibronectin with identical affinity, thus facilitating the study of antibody derivatives both in syngeneic immunocompetent mouse models of cancer and in human tumors. The F8 antibody selectively localizes to tumor blood vessels in vivo, as revealed by quantitative biodistribution studies with radioiodinated protein preparations and by ex vivo microautoradiographic analysis (37). The antibody retains its tumor homing properties when used in various formats (e.g., diabody, small immune protein and IgG), with a residence time of several days in the tumor extracellular matrix (33).

Interleukin-2 (IL2) is a pro-inflammatory cytokine which induces the activation and proliferation of T cells and NK cells in vivo. Recombinant human IL2 is a marketed anti-cancer product, which can cure patients with metastatic melanoma or with kidney cancer (38-41), albeit at the expense of severe toxicities. Several antibody-IL2 fusion proteins have been tested in mouse models of cancer and some have progressed to clinical trials in patients with solid malignancies (9, 42) or acute leukemias (36). The F8-IL2 fusion protein has previously exhibited a potent anti-tumor activity in various mouse models of cancer (2, 8, 36, 43), which can be potentiated by the addition of other therapeutic agents. Importantly, the F8 antibody has also been shown to act as a convenient pharmacodelivery agent for highly-potent cytotoxic drugs (e.g., the DM1 maytansinoid, a microtubular inhibitor), when equipped with a suitable linker for cleavage at the site of disease (44). A combination of F8-IL2 with F8-based antibody-drug conjugates mediated a potent anti-cancer activity, leading to complete eradication of C1498 tumors in immunocompetent mice. The process was found to be dependent both on the action of CD8+ T cells and of NK cells, as revealed by antibody-based immunodepletion studies (27).
In this article, we describe a novel class of trifunctional armed antibody products (termed “immunocytokine drug conjugate”, or IDC), which can be efficiently prepared with high biochemical purity and with full retention of functional activity of the three moieties present in the molecule (i.e., antigen binding, cytokine activity and drug-mediated cytotoxic activity upon linker cleavage). In particular, the F8-IL2-SS-DM1 IDC product, featuring the simultaneous presence of IL2 and disulfide-linked DM1 drug on a recombinant F8 antibody scaffold in diabody format, selectively localized to solid tumors after intravenous administration and mediated a potent anti-cancer activity in two immunocompetent mouse models (F9 and C1498).
Materials and Methods

Construction of immunocytokine. The F8-IL2-SH and F8-IL2 immunocytokines were genetically assembled by successive PCR assembly steps, in analogy to previously published procedures (8) and cloned into pcDNA3.1 expression vector (Invitrogen) downstream of a mammalian secretion signal sequence. In the case of F8-IL2-SH one extra amino acid (Cysteine) was introduced into the 21aa linker, yielding a 22aa linker to obtain one free thiol group on the cysteine side chain for site specific drug coupling reactions. The complete gene sequences for the F8-IL2-SH, F8-IL2 and F8-SH products can be found in the Supplementary Data.

Cell lines and mice. Chinese Hamster Ovary cells (CHO-S, obtained from Invitrogen, 2007) cells were used for the production of recombinant proteins. The tumor cell lines used for cytotoxicity assays and therapy studies were the murine teratocarcinoma cell line F9 [CRL-1720, obtained from American Type Culture Collection (ATCC), 2003], the murine AML cell line C1498 [TIB-49, obtained from American Type Culture Collection (ATCC), 2008], the murine B-cell lymphoma cell line A20 [TIB-208, obtained from American Type Culture Collection (ATCC), 2008] and the murine melanoma cell line K1735M2 which was a kind gift of Prof. Silvio Hemmi (University of Zürich). For T-cell activation experiments the murine cytotoxic T-cell line CTLL2 was used [TIB-214, obtained from American Type Culture Collection (ATCC), 2004]. CHO-S cells in suspension were cultured in shaker incubators (37 °C) in PowerCHO-2CD medium (Lonza, Switzerland) containing 8 mM Ultraglutamine (Lonza, Switzerland) and HT supplement (Lonza, Switzerland). F9 cells were cultured on 0.1% gelatin-coated tissue flasks or wells in DMEM (Thermo Scientific, USA) supplemented with 10% FCS (Fetal Calf Serum, Thermo Scientific, USA) and Antibiotic-Antimyctic (Thermo Scientific, USA) at 37 °C and 5% CO2. C1498 cells were cultured in DMEM supplemented with 10% FCS, 1 mM Sodium Pyruvate (Thermo Scientific, USA) at 37 °C and 5% CO2. A20 cells were cultured in RPMI-1640 (Thermo Scientific, USA) with 10% FCS, 0.05 mM beta-mercaptoethanol (Thermo Scientific, USA) and Antibiotic-Antimyctic at 37 °C and 5% CO2. K1735 cells were cultured in DMEM with 10% FCS, 1 mM Sodium Pyruvate (Thermo Scientific, USA) and Antibiotic-Antimyctic at 37 °C and 5% CO2. CTLL2 cells were cultured at 37 °C and 5% CO2 in RPMI-1640 medium (Thermo Scientific, USA) supplemented with 10% FCS, Antibiotic-Antimyctic, 1 mM Sodium Pyruvate, 50μM beta-mercaptoehntanol, human IL2 (20 units/ml) (Sino Biological, China) and 1%v/v
Ultralglutamine. Female 129/SvFv mice and female C57BL/6J mice were obtained from Charles River (Germany). Cell lines were obtained from Invitrogen or ATCC cell bank and passaged in our laboratory according to the recommended protocols. No further authentication was performed.

**Protein expression and purification.** Immunocytokines were expressed using transient gene expression procedures (45). For 200mL of production 200.10^6 CHO-S cells were resuspended in 100mL ProCHO4 (Lonza). Plasmid DNA (250 μg) was mixed with 150 mM NaCl to reach a final volume of 5mL. 5mL of 250 mg/ml 25-kDa linear polyethylene imine (PEI; 1 mg/mL solution in water at pH 7.0; Polysciences) in 150 mM NaCl were added to the DNA/NaCl solution and allowed to stand at room temperature for 10 minutes. The solution containing the PEI–DNA complexes was then added to the cells and gently mixed. The transfected cultures were incubated in a shaker incubator at 37°C. At 4 hours after transfection, the culture was diluted with 100 mL Power-CHO-2CD medium and then incubated at 31°C in a shaking incubator for 6 days. The fusion proteins were purified from the cell culture medium by protein A affinity chromatography and then dialyzed against phosphate buffered saline (PBS, 6 mg/ml NaCl, 3.12 mg/ml NaH₂PO₄, 5.34 mg/ml Na₂HPO₄, 300 mM NaOH, pH7.4).

**Conjugation of protein to small molecules.** Conjugation of fusion proteins to small molecule drug (DM1) was performed as previously described by our group (46). Briefly, the proteins in saline solution were reduced under mild conditions with 30 molar equivalents tris(2-carboxyethyl)phosphine (TCEP) three hours at room temperature. Free thiol groups were reacted 40 min at room temperature with 1000 molar equivalents 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) added to the reduced protein solution. Excess DNTB, which was not covalently bound to protein, was removed by buffer exchange column (PD-10, GE Healthcare) according to manufacturer’s protocol for μg scale production or by HiTrap (GE Healthcare) columns using an FPLC pump (Äkta Purifier, GE) for mg scale production. The coupling buffer was 150 mM phosphate buffered saline (PBS) pH7.4, containing 5% w/v sucrose and 10% N,N-dimethylacetamide (DMA) (Acros Organics). Subsequently Protein-DTNB conjugates were reacted with 15 molar equivalents DM1-SH for 5min at room temperature and the reaction was quenched with 500 molar equivalents iodoacetamide for 10min at room temperature. Excess DM1 and iodoacetamide were removed in analogy to the procedure described for Protein-DTNB
conjugates. Characterization of conjugate was performed on an ÄKTA Pure FPLC system with Superdex200 10/300GL column (size exclusion) and mass spectrometry (ESI-Quadrupole).

**Surface plasmon resonance.** Protein solutions were filtered (using 0.22 μm PVDF filters) and their binding properties analyzed using a BIACore3000 instrument (BIACore) and an antigen coated chip, which was prepared by covalent coupling of bacterially produced EDA to a CM5 sensor chip (GE Healthcare) according to the manufacturer’s protocol.

**Cytotoxicity assay.** Tumor cell lines were seeded at different densities in 96 well plates to achieve a starting confluency of approximately 10-20%. The optimal seeding cell densities for different cell types were determined by light microscopic monitoring of confluence and growth rate as follows: 10,000 cells/well for A20, 8000 cells/well for C1498, 6000 cells/well for F9, 10,000 cells/well for K1735. All cell types were grown for one week prior to the experiment to ensure stable growth and seeded in triplicates in 96 well plates at 37°C 5%CO2. After 24h different dilutions of drugs in culture medium containing 0.3% DMSO were added to the cells and cultures were incubated for 72h at 37°C 5% CO2. After 72h 20μl MTS solution (CellTiter96® AQueous One Solution, Promega, USA) was added to each well, cultures were incubated at 37°C 5% CO2 for 24h and absorption at 490nm of each well was measured on a Molecular Devices SpectraMax® Paradigm® spectrophotometer.

**Cell proliferation assay.** CTLL2 cells were grown for two weeks in fully supplemented growth medium with a maximum density of 10^5 cells/ml. Cells were then starved of IL2 by washing away old growth medium and culturing them in medium lacking IL2 for 48h. Starved cells were seeded in 96 well plates at 2.10^4 cells/well and incubated with different dilutions of antibody-drug conjugate or unconjugated IL2 (Proleukin®, Aldesleukin®, Novartis, Switzerland) for 24h at 37°C 5%CO2. After 24h 20μl MTS solution (CellTiter96® AQueous One Solution, Promega, USA) was added to each well, cultures were incubated at 37°C 5% CO2 for 24h and absorption at 490nm of each well was measured on a Molecular Devices SpectraMax® Paradigm® spectrophotometer.

**Biodistribution studies.** Tumor-bearing mice were obtained by subcutaneous injection of F9 teratocarcinoma cells (10^6) (129Sv mice) or C1498 AML cells (2.10^6) (C57BL/6J mice) in the flank of 15-18 weeks old female mice. Five days after tumor implantation, 10 μg (0.09 nmol) ^125I radiolabelled F8-IL2-SS-DM1 and F8-IL2 were injected into the lateral tail vein of the animals.
Immunoreactivity of the antibody preparation after radioiodination was confirmed by binding to EDA resin. Mice were sacrificed 24 hours after injection. Organs were weighed and radioactivity was counted using a Packard Cobra gamma counter, without prior perfusion. Radioactivity content of representative organs was expressed as the percentage of the injected dose per gram of tissue (%ID/g ±SE).

Therapy experiments. F9 teratocarcinoma cells (10^6) (129Sv mice) or C1498 AML cells (2.10^6) (C57BL/6J mice) were injected subcutaneously into the flank of 15-18 weeks old female mice. Five days after tumor implantation, when tumors were clearly palpable, mice were randomized in different treatment groups. Therapy was carried out by intravenous injection into the tail vein. 129Sv mice were injected 4 times, every 48 hours with 50 μg protein or saline (PBS), C57BL/6 mice were injected three times, every 48 hours with 10 μg or 30 μg protein or saline. End-point criteria for the experiments were a tumor size of 2000cm³ or observed weight loss of more than 15% (toxicity threshold). Mice that had been rendered tumor-free for at least 40 days were considered cured. Experiments were carried out under a project license granted by the Veterinaeramt des Kantons Zuerich, Switzerland.

Unit conversion. The conversion of maximum tolerated dose (MTD) values was done using the following formula: 
\[
\text{Body surface (m}^2\text{)} = \sqrt{\left(\text{height (cm)} \times \text{weight (Kg)}\right)/3600}
\]
and under the assumption that the average patient has a weight of 80kg and a height of 180cm.
Results

Choice of DM1 as a cytotoxic payload for drug conjugates
We assessed the in vitro cytotoxic potential of two potent microtubular inhibitors (cemadotin and DM1), equipped with a thiol group suitable for protein coupling or with a methylthioether moiety [Figure 1a]. Both cemadotin and DM1 derivatives have previously been used for the preparation and in vivo testing of ADCs, based on the F8 antibody (44, 46, 47). Four murine cancer cell lines were used in the assay (F9, A20, C1498 and K1735), which we have previously shown to reproducibly grow solid tumors in immunocompetent recipient mice (36, 48, 49). Those tumors contained blood vessels, which strongly stained for EDA expression using the F8 antibody [Figure S1]. In the cell lines, DM1 derivatives consistently exhibited a more potent cytotoxic activity, compared to cemadotin derivatives [Figure 1b]. In line with previous reports (19), S-protected thiol drugs led to an increased anti-cancer activity, but DM1-SH exhibited an acceptable cytotoxic potential and was chosen as payload for the preparation of drug conjugates.

Preparation and in vitro characterization of chemically-defined Immunocytokine-Drug Conjugates
The F8-IL2 immunocytokine was chosen as the therapeutic protein for drug coupling experiments, as it had previously been shown to selectively localize to tumor blood vessels in vivo and to display a potent anti-cancer activity in immunocompetent mouse models of the disease (2, 8, 36, 43). We used the F8 antibody in scFv format (50) with a 5-aminoacid linker between VH and VL domains, driving the formation of a non-covalent homodimeric “diabody” structure (51) [Figure S2]. The antibody was genetically fused to recombinant human interleukin-2 (IL2), using a polypeptide linker with a cysteine residue (F8-IL2-SH), suitable for subsequent chemical modification strategies, or with no cysteine residue (F8-IL2) [Figure 2a]. Both proteins could be expressed in mammalian cells and purified to homogeneity. In non-reducing conditions, F8-IL2-SH exhibited the formation of a disulfide-linked homodimeric product, which could however be quantitatively reduced [Figure 2b]. In addition, a variant of the F8 diabody (termed F8-SH), lacking the IL2 payload but featuring an N-terminal free cysteine residue, was expressed and purified from CHO cells. F8-IL2-SH and F8-SH were site-specifically conjugated to DM1-SH using a chemical procedure based on Ellman’s reagent, previously described by our group (46) [Figure 2c]. All products, which were characterized by
SDS-PAGE, gel filtration and mass spectrometry analysis, were shown to be pure and homogenous [Figure 2d].

The ability of the F8-IL2 fusion protein and of the corresponding F8-IL2-SS-DM1 drug conjugate to recognized the cognate antigen was confirmed by ELISA (not shown) and by BIAcore analysis, revealing comparable EDA binding profiles [Figure 3a]. In addition, the IL2 moiety displayed comparable biological activity in commercially available recombinant IL2 (Proleukin®, Aldesleukin®), in F8-IL2 and in F8-IL2-SS-DM1 [Figure 3b].

**In vivo** tumor-targeting activity and anti-cancer properties of Immunocytokine-Drug Conjugates

The ability of F8-IL2-SS-DM1 to preferentially accumulate in solid tumors, following intravenous administration, we tested in mice bearing subcutaneously-grafted F9 or C1498 tumors, using radioiodinated protein preparations. Twenty-four hours after intravenous administration, both F8-IL2 and F8-IL2-SS-DM1 exhibited comparable biodistribution profiles, with a preferential product accumulation in the tumor [Figure 4]. Some accumulation of the products was also observed in the kidney, in line for what had previously been reported for F8-IL2 in C1498 tumors (36) and with the renal clearance mechanism. The same tumor models were used for the study of anti-cancer therapeutic activity.

Immunocompetent mice bearing C1498 AML chloromas were treated with three injections of 10 µg of F8-IL2, F8-SS-DM1 or a combination of the two agents. Additionally, one group of mice was treated with 10 µg doses of the F8-IL2-SS-DM1 IDC product, while a control group received injections of saline solution. At these doses, F8-SS-DM1 exhibited only a modest anti-tumor activity, while F8-IL2 potently inhibited tumor growth. The therapeutic action of this cytokine could be potentiated either by combination with F8-SS-DM1 or by incorporating both the IL2 and the DM1 moiety in the same product (F8-IL2-SS-DM1) [Figure 5a]. None of the study groups exhibited a body weight loss greater than 5% [Figure 5b]. The experiment was repeated at higher doses (30 µg), confirming a strong anti-cancer activity of F8-IL2-SS-DM1 [Figure 5 c,d]. Therapeutic results obtained at 10 and 30 µg doses were similar. It is difficult to directly compare therapeutic data obtained in different sets of mice and different dates, as tumor growth rate and vascularity can slightly change across experiments.
Similar experiments were performed in Sv129Ev mice bearing subcutaneously-grafted murine F9 teratocarcinomas at low (30μg) and high (50μg) doses, which revealed a potent anti-tumor activity of the IDC product while none of the study groups exhibited a body weight loss greater than 10% [Figure 5 e-h].
Discussion

We have produced and characterized a novel class of armed antibody products ("immunocytokine drug conjugate", or IDC), which combine a tumor-targeting antibody moiety and two therapeutic payloads in a single molecular entity. We implemented the new methodology by installing the immunostimulatory cytokine IL2 and the potent cytotoxic maytansinoid DM1 onto the clinical-stage F8 antibody. The resulting trifunctional biopharmaceutical exhibited a potent anti-tumor activity in two syngeneic murine models of cancer. Importantly, all functional moieties of the IDC retained their biological activity and the product was able to selectively localize to tumors in vivo.

In the syngeneic C1498 mouse model of AML chloromas, a potent therapeutic activity could be observed at very low doses of F8-IL2-SS-DM1 (0.5 mg/kg), including some mice which were cured [Figure S3]. This observation is encouraging, since the IDC product appears to be active at substantially lower doses, compared to the ones used for the corresponding F8-SS-DM1 ADC products (44). Cost-of-goods may limit the industrial development of certain ADC products and their conversion into IDC format may represent a valuable avenue for the improvement of anticancer activity. Importantly, the antibody-mediated delivery of IL2 is potently active not only in mouse models of AML, but also in patients (36).

The molecular and cellular mechanisms for the observed synergy are still unknown, but we speculate that the cytotoxic damage, induced by DM1, may contribute to an increased tumor immunogenicity and vascular permeability, as recently reported in other studies (2).

IDCs represent one class of multi-specific antibody-based therapeutic agents. The combination of two binding specificities in the same antibody molecule has long attracted the interest of immunologists. BiTEs (bispecific T-cell engagers) and DART (Dual Affinity Re-Targeting platform) are representative examples of bispecific antibody products in clinical development (52, 53). Alternatively, the implementation of two cytokine payloads (1) or of two cytotoxic agents onto the same antibody molecule has also been proposed.

In principle, the IDC strategy should be broadly applicable to a variety of antibodies, cytokines and cytotoxic agents. In practice, however, it will be important to combine payloads of
comparable activity into the same IDC product, in order to exploit the optimal potential of this technology. The maximal tolerated dose of cytokines in patients may vary substantially (e.g. 9.4-132 μg/Kg for IL2 per day (54), 1 μg/Kg for IL12 per day (55, 56), 4 μg/Kg for TNF per day (57-59), 0.5 μg/Kg for GM-CSF per day (60), 0.46-0.95 μg/Kg per day for IFNa (60, 61), 1-20 μg/kg per day for IL10 (62), 178 μg/Kg per day for IFNb (63). Similarly, the cytotoxic agents which have been used for ADC development range from doxorubicin and methotrexate, with \textit{in vitro} cell killing potency in the 0.1-1 μM range (64) to pyrrolobenzodiazepines which are able to kill tumor cells \textit{in vitro} at sub-picomolar concentrations (65). Should the cytokine payload be more active compared to the cytotoxic agent, it would be conceivable to fine-tune the corresponding biological activity by amino acid mutations. Indeed, there is growing evidence that certain cytokine mutants (e.g. the IL2 “superkine” bearing mutations in five amino acid residues) may acquire beneficial properties, such as improved proliferation induction potential of T-cells irrespective of the presence of the normally essential high affinity alpha subunit of IL2 receptor, more potent induction of downstream phosphorylation signal improved \textit{in vivo} anti-tumor activity and reduced expansion of regulatory T cells (66).

The combined use of immunostimulatory cytokines and cytotoxic compounds may be counterintuitive, as the anti-cancer drug could kill leukocytes \textit{in vivo} and therefore inhibit cytokine activity. However, there is growing evidence that the mechanism of action of anti-cancer cytotoxic drugs may be more complex. For example, certain chemotherapeutic agents have been shown to significantly potentiate the anti-tumor effect of immunocytokines. Specifically inefficient extravasation of drug and immune cells towards to site of disease is a major obstacle for efficacious targeted therapy, which can be overcome by pretreatment with chemotherapeutic agents. Paclitaxel increases vascular permeability in tumors (2) and reduces interstitial fluid pressure (16, 17). These mechanisms lead to enhanced infiltration and activation of leukocytes (2, 18) and provide a rationale for the combined use of cytotoxic small molecule drugs and cytokines.

Cytotoxic drugs may exert immunostimulatory effects by influencing the functions of effector cells of the immune system, by lymphodepletion or subversion of immunosuppressive mechanisms (5). For example, gemcitabine-induced apoptosis in established tumors enhances dendritic cell-dependent cross-presentation of tumor antigens (67, 68), increasing the sensitivity of tumor cells to lytic killing by cytotoxic T cells (69, 70). Both gemcitabine and
cyclophosphamide has been shown to reduce the number and inhibitory function of Tregs (71-73).

In practice, the identification of the recommended dose for pharmacological treatment will require the study of activity and tolerability at multiple dose levels. In the field of cancer immunotherapy, the implementation of larger Phase I clinical trials, with a sufficient number of patients in each cohort for the determination of therapeutic activity, is becoming more and more common. For instance, two recent examples of remarkable results in clinical melanoma treatment obtained with the use of anti-PD-1 antibodies (Lambrolizumab from Merck and Nivolumab by Bristol-Meyer-Squibb) relied on large cohorts of patients at each dose level, in Phase I studies (74, 75).

It is well established that the pharmacotherapy of cancer relies, in most cases, on combination therapy. The development of multifunctional products, such as the IDCs described in this article, should avoid the parallel clinical development of individual agents, which may lack sufficient activity when used as monotherapy. The traditional “one-at-a-time” development of anti-cancer drugs is not only costly and time-consuming, but may also deny patients the option of a potentially efficacious treatment. In particular, the chemically-defined incorporation of an IL2 moiety and of a potent cytotoxic agent into a single biopharmaceutical agent holds promises for the therapy of AML, since ADCs and IL2-based immunocytokines have already exhibited promising activity both in animal models and in cancer patients (6, 7, 14, 15, 21, 42, 76-79).
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References


Figure Captions

Figure 1 The F9 and C1498 murine tumor cell lines are more susceptible to DM1 than Cemadotin. a) Cemadotin and DM1 cytotoxic drug and derivatives containing either a free thiol (Cem-CH2-SH, DM1-SH) or an alkyl group (Cem-CH2-Me, DM1-SMe). b) Dose dependent percent viability (72h endpoint measurement) of four murine cell lines in vitro with different concentrations of DM1-SH (open circle), DM1-SMe (filled circle), Cem-CH2-SH (open triangle) and Cem-CH2-SMe (filled triangle) as measured my MTS colorimetric assay. Viability is given as a percentage of the absorption signal (490nm) of control cells incubated with culture medium alone.
Figure 2 Biochemical characterization of the F8-IL2-SS-DM1 immunocytokine-drug-conjugate. a) Genetic construct for F8-IL2-SH. Sp, post-translationally cleaved excretion signal peptide; F8VH, variable domain heavy chain; F8VL, variable domain light chain; 5aa, short 5 amino acid linker; 21aaCys, long 22 amino acid linker containing cysteine; hIL2, human IL2. b) PAGE analysis of F8-IL2-SH and F8-IL2 expressed in CHO cells and purified by ProteinA affinity chromatography. Reducing conditions with beta-mercaptoethanol (lane 1 and 3) and non-reducing conditions (lane 2 and 4). c) Conjugation of proteins containing a free cysteine to DM1-SH. Protein is reduced with TCEP, activated with DTNP (Ellmann’s reagent) and conjugated to DM1-SH by disulfide bridge formation. d) Profiling of F8-IL2-SH, F8-IL2-SS-DM1 and F8-SS-DM1 by gel filtration (GE S200 10/300GL, Äkta Pure) and MS (ESI, Quadrupole). Elution profiles show the expected retention volumes and MS shows the expected masses of unconjugated and conjugated proteins: 42016.6 Da (calculated) and 42020 Da (measured) for the F8-IL2-SH monomer; 42754.9 Da (calculated) and 42760 Da (measured) for the F8-IL2-SS-DM1 monomer.

Figure 3 F8-IL2-SS-DM1 is biologically active in vitro. a) Surface plasmon resonance (Biacore) analysis of difference concentrations of F8IL2, F8-IL2-SS-DM1 and F8-SS-DM1 on a CM5 chip coated with soluble EDA antigen. b) T-cell stimulatory properties of the IL2 moiety of antibody drug conjugates was benchmarked against commercially available IL2. Dose dependent percent proliferation (48h endpoint) of the CTLL2 murine cell line in vitro with different concentrations of F8-conjugates was measured by MTS colorimetry. Proliferation is given as a percentage of the absorption signal (490nm) of control cells incubated with culture medium alone. F8-IL2-SS-DM1, DM1 conjugate; F8-IL2-SS-Cem, cemadotin conjugate; F8-IL2-SS-Glut, glutathion conjugate.

Figure 4 F8-IL2-SS-DM1 selectively accumulates at the tumor site. Immunocompetent mice bearing F9 or C1498 syngeneic tumors were injected with 10 µg (0.09 nmol) 125I radiolabelled a) F8-IL2-SS-DM1 or b) F8-IL2. Bars show the mean ±SD percentage injected dose per gram of tissue (%ID/g). Immunoreactivity after radiiodination was confirmed by binding to EDA resin (data not shown).

Figure 5 F8-IL2-SS-DM1 matches the anti-tumor effect of combination therapy and shows improved tumor retardation over the single-payload immunocytokine F8-IL2.
experiments were performed twice for each tumor model with increasing dosage. a) Mean tumor volume ±SD (mm³) is shown over time in 129Sv mice (n=4 per treatment group) implanted s.c. with F9 tumor cells. Mice were injected i.v. in the tail vein with 10 μg conjugate, immunocytokine or saline every 48h for 3 injections in total (arrows). b) Mean weight change is given in percent of weight at the start of therapy for each treatment group. Mice were weighed every day. c) Mean tumor volume ±SD (mm³) is shown over time in C57BL/6 mice (n=5 per treatment group) implanted s.c. with C1498 tumors. Mice were injected i.v. in the tail vein with 30 μg respectively of conjugate, immunocytokine or saline every 48h for 3 injections in total (arrows). d) Mean weight change is given in percent of weight at the start of therapy for each treatment group. Mice were weighed every day. e) Mean tumor volume ±SD (mm³) is shown over time in 129Sv mice (n=3 per treatment group) implanted s.c. with F9 tumors. Mice were injected i.v. in the tail vein with 30 μg respectively of conjugate, immunocytokine or saline every 48h for 4 injections in total (arrows). f) Mean weight change is given in percent of weight at the start of therapy for each treatment group. Mice were weighed every day. g) Mean tumor volume ±SD (mm³) is shown over time in 129Sv mice (n=5 per treatment group) implanted s.c. with F9 tumors. Mice were injected i.v. in the tail vein with 50 μg respectively of conjugate, immunocytokine or saline every 48h for 4 injections in total (arrows). h) Mean weight change is given in percent of weight at the start of therapy for each treatment group. Mice were weighed every day.
Figure 1

(a) Structures of DM1-SH and DM1-SMe, with R = H and R = CH₃, respectively. Cem-CH₂-SH and Cem-CH₂-SMe, with R = CH₂SH and R = CH₂SCH₃, are also shown.

(b) Viability graphs for different cell lines (F9, A20, C1498, K1735) showing the effect of DM1-SH, DM1-SMe, Cem-CH₂-SH, and Cem-CH₂-SMe at various concentrations (M) on cell viability.
Figure 2

a) [Diagram showing HindIII, Sp, F8V, 5aa, F8V, 21aaCys, hIL2, pTLF8CysIL2]

b) [Image of gel showing F8-IL2-SH and F8-IL2]

c) [Diagram showing 1. TCEP reduction, 2. DTNB, DM1-SH, S-DM1, DM1-S]

1. TCEP reduction
2. DTNB

1. TCEP reduction
2. DTNB

1. TCEP reduction
2. DTNB

1. TCEP reduction
2. DTNB

1. TCEP reduction
2. DTNB

1. TCEP reduction
2. DTNB

1. TCEP reduction
2. DTNB

1. TCEP reduction
2. DTNB

1. TCEP reduction
2. DTNB
Figure 4

(a) S—DM1

(b) F9 (24h)

C1498 (24h)

%ID/g

Tumor | Liver | Lung | Spleen | Heart | Kidney | Intestine | Blood | Stomach

F9 (24h)

C1498 (24h)
Figure 5

a) Tumor volume (mm$^3$) over time (days) for different groups:
- F8-IL2-SS-DM1
- F8-IL2
- F8-SS-DM1
- Combination
- Saline

b) Weight change (%) over time (days) for different groups:
- F8-IL2-SS-DM1
- F8-IL2
- F8-SS-DM1
- Combination
- Saline

Tumor volume (mm$^3$) and weight change (%) graphs for F8-IL2-SS-DM1, F8-IL2, F8-SS-DM1, Combination, and Saline groups.

(C1498) and (F9) designations indicate specific conditions or groups.

Toxicity threshold marked on the graphs.
Molecular Cancer Therapeutics

A chemically-defined trifunctional antibody-cytokine-drug conjugate with potent anti-tumor activity

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