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Non-internalizing antibody-drug conjugates display potent anti-cancer activity upon proteolytic release of monomethyl auristatin E in the sub-endothelial extracellular matrix

Rémy Gébleux¹, Marco Stringhini¹, Ruben Casanova², Alex Soltermann², and Dario Neri^{1,*}

¹Department of Applied Biosciences, Swiss Federal Institute of Technology (ETH Zürich), Vladimir-Prelog-Weg 4, CH-8093 Zürich (Switzerland) ²Institute of Pathology, University Hospital Zurich, Schmelzbergstrasse 12, CH-8091 Zurich (Switzerland)

Abstract

Antibody-drug conjugates (ADCs) represent a promising class of biopharmaceuticals with the potential to localize at the tumor site and improve the therapeutic index of cytotoxic drugs. While it is generally believed that ADCs need to be internalized into tumor cells in order to display optimal therapeutic activity, it has recently been shown that non-internalizing antibodies can efficiently liberate disulfide-linked drugs at the extracellular tumor site, leading to potent anticancer activity in preclinical animal models. Here, we show that engineered variants of the F16 antibody, specific to a splice isoform of tenascin-C, selectively localize to the sub-endothelial tumor extracellular matrix in three mouse models of human cancer (U87, A431, MDA-MB-231). A site-specific coupling of F16 in IgG format with a monomethyl auristatin E (MMAE) derivative, featuring a valine-citrulline dipeptide linker equipped with a self-immolative spacer, yielded an ADC product, which cured tumor-bearing mice at a dose of 7 mg/Kg. The observation of an efficient extracellular proteolytic cleavage of the valine-citrulline linker was surprising, as it has generally been assumed that this peptidic structure would be selectively cleaved by cathepsin B in intracellular compartments. The products described in this article may be useful for the treatment of human malignancies, as their cognate antigen is strongly expressed in the majority of human solid tumors, lymphomas and aggressive leukemias, while being virtually undetectable in most normal adult tissues.

Keywords

Vascular targeting; antibody-drug conjugates; IgG; SIP; MMAE; A1 domain of tenascin-C

Introduction

The majority of cytotoxic agents used for the pharmacotherapy of cancer do not preferentially localize at the tumor site, causing undesired toxicities which prevent dose

*Corresponding Author [Tel: +41-44-6337401; neri@pharma.ethz.ch].

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escalation 1–4. Antibodies capable of selective localization at the tumor site may serve as pharmacodelivery vehicles, with the potential to improve the therapeutic index of anticancer drugs 5–8. Two ADC products (Adcetris™ and Kadcyla™) have gained marketing authorization for the treatment of certain types of cancer. Adcetris™ features the use of an anti-CD30 IgG coupled to monomethyl auristatin E (MMAE) through a linker comprising a valine-citrulline dipeptide followed by a self-immolative spacer, while Kadcyla™ consists of the anti-HER2 trastuzumab antibody coupled to the maytansinoid DM1 with a stable heterobifunctional cross-linker 9, 10.

It is generally believed that efficacious ADC products would require the ability to selectively bind and internalize into the cognate tumor cells 5–8. This strict requirement, however, has been questioned. For example, it was observed that anti-lymphoma ADC products worked well in mouse models of cancer also with antigens which were poorly internalized (e.g., CD20, CD21, CD22) 11. Researchers in Japan had proposed an attractive tumor targeting strategy, using antibodies specific to fibrin or to collagen 4 12. This approach, termed “Cancer stromal targeting (CAST)” relied on the observation that blood coagulation and the subsequent fibrin deposition generates a provisional stroma, which is frequently observed in many solid malignancies 12. Non-internalizing ADCs were generated, which were potentially active in certain mouse models of cancer 13, 14.

We have reported that non-internalizing ADC products, based on monoclonal antibodies specific to splice-isoforms of fibronectin, could liberate their payload in the tumor sub-endothelial extracellular space and display a potent therapeutic activity 15–17. In those cases, cytotoxic drugs were coupled to the antibody via disulfide linkers, which could be cleaved at the site of disease by reducing agents. The progressive death of tumor cells releases increasing amounts of glutathione (the most abundant thiol in the intracellular space) 18, 19, leading to an amplification of the drug release process 20. Interestingly, a high variability in the stability of disulfide-linked payloads, which impacted on therapeutic performance *in vivo*, was observed when comparing ADC products based on intact antibodies and smaller antibody fragments 17.

In principle, it would be desirable to develop non-internalizing ADC products with peptidic linkers and a variety of cytotoxic payloads. Certain peptidic linkers (e.g., those incorporating the valine-citrulline moiety) have been reported to be stable in circulation *in vivo*, thus contributing to a benign safety profile 21. For example, the linker-payload used in the approved pharmaceutical Adcetris™ contains a maleimidocaproyl (mc) spacer, a protease sensitive dipeptide valine-citrulline (vc), a self-immolative spacer, para-amino benzylcarbonyl (PAB) and finally the antimitotic monomethyl auristatin E (MMAE) 9. Upon binding to tumor cell surface antigens, the ADCs can internalize, traffic to lysosomes and be metabolized by lysosomal proteases (such as Cathepsin B) to release the free payload (MMAE) 22, 23.

Tenascin-C splice variants represent a group of non-internalizing antigens, which can be efficiently targeted by cognate monoclonal antibodies *in vivo* 24–26. In particular, the A1 domain of tenascin-C can be targeted by the fully-human F16 antibody, which has exhibited promising biodistribution results in mouse models of cancer 27 and in patients 28, 29. The

F16 antibody strongly stains the majority of human solid tumors 30, lymphomas 31 and acute leukemias 32, while a weak staining of normal adult tissues can only be observed in placenta and in the endometrium in the proliferative phase 31.

In this article, we report that the binding properties and the tumor targeting performance of the F16 antibody could be improved by substitution of an asparagine residue in the VL domain with a glutamine, which abolished N-linked glycosylation. The F16 mutant was expressed in engineered human IgG1 and as small immune protein (SIP) 27, 33, 34 formats, which contained a unique cysteine residue for drug coupling 17, 35. The antibodies were coupled to the linker payload of Adcetris™, yielding ADC products which selectively localized at the tumor site and led to cancer cures in mouse models of human tumors. The results indicate that not only reducing agents, but also extracellular proteases, can be conveniently used for an efficient drug release at the tumor site, with promising therapeutic activity.

Materials and Methods

Cell Culture: cell lines, incubation and manipulation conditions

Transfected CHO-S cells (Invitrogen) were cultured in suspension in PowerCHO-2CD medium (Lonza), supplemented with Ultraglutamine-1 (Lonza), HT-supplement (Gibco) and Antibiotic-Antimycotic (Gibco). A-431 epidermoid carcinoma cells (ATCC, CRL-1555) and MDA-231-MB (ATCC, HTB-26) were grown in DMEM (Gibco) while U-87 glioblastoma cells (ATCC, HTB-14) were grown in MEM (Gibco). All media were supplemented with 10% FBS (Gibco) and Antibiotic-Antimycotic (Gibco) and incubated at 37 °C in 5% CO₂ atmosphere.

Animals and tumor models

Ten to twelve weeks old female Balb/c nude mice were obtained from Janvier Laboratories (France). A431 cells (2.8×10^6), U87 (6×10^6) or MDA-231-MB (15×10^6) were implanted subcutaneously in the flank. Animals were sacrificed when tumors volumes reached a maximum of 2000 mm³ or weight loss exceeded 15%. Experiments were performed under project licenses issued by the Veterinäramt des Kantons Zürich, Switzerland (Bew. Nr. 42/2012 and Nr. 027/15)

Cloning, expression and protein characterization

The gene structure for SIP(F16), its cloning, expression and characterization have previously been described 27. The F16 antibody in IgG format was cloned and expressed in CHO-S cells (Invitrogen) using a previously described strategy 36, except for the cysteine residues of the heavy chain in position 220, 226 and 229, which were mutated into serine residues. In IgG(F16)*, the Asparagine 88 residue which was part of a consensus glycosylation sequence (NSS) was mutated into Glutamine. The full aminoacid sequences for SIP(F16), IgG(F16) and IgG(F16)* can be found in Supplementary Figure S1.

Antibody-MMAE conjugates preparation

The antibody in SIP or IgG format was reduced with 30 equivalents (calculated on the basis of antibody monomers, each containing a single cysteine residue) of tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl, ACBR) in phosphate buffer saline (PBS, p.H. = 7.4). The reduced protein was purified by size exclusion chromatography on an HiPrep 26/10 Desalting column (GE Healthcare); 1mM DTPA (Sigma-Aldrich)/PBS was used as a mobile phase at a flow rate of 1.5-2 mL/min. The recovered protein was pooled and concentrated using Vivaspin® Turbo 15 (Sartorius) in order to remain in the capacity limit of the FPLC-loop. Ten equivalents of Vedotin (MC-vc-PAB-MMAE, Concortis Biosystems) were dissolved in DMSO (Sigma-Aldrich) and added to the reduced protein with a final DMSO content of 5% (v/v). IgG and Vedotin were let react under stirring for 15' at RT, the reaction was then quenched with L-Cys (Fluka) at a final concentration of 1 mM for 10' at RT. Final product was FPLC-purified as described previously. The ADCs were then formulated at the desired concentration by centrifugation using Vivaspin devices (Sartorius), snap-frozen in liquid nitrogen and stored at -80 °C until further use.

Characterization of ADC products

All ADC products were analyzed by SDS-PAGE (Invitrogen), size exclusion chromatography (Superdex200 10/300GL, GE Healthcare) and protein mass spectrometry. Liquid chromatography-mass spectrometry (LC-MS) was performed on a Waters Xevo G2-XS Qtof instrument (ESI-ToF-MS) coupled to a Waters Acquity UPLC H-Class System using a 2.1 x 50 mm Acquity BEH300 C4 1.7 µm column (Waters). 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) were used as mobile phase at a flow rate of 0.4 mL/min. Gradient was programmed as follows: after 1.5 min isocratic with 95% solvent A, stepwise change from 95% solvent A to 95% solvent B in 4.5 min (10% increase every 0.5 min), back to 95% solvent A in 0.5 min, linearly to 95% solvent B and back to 95% solvent A in 2.25 min (last step repeated twice). The binding properties of SIP(F16)-MMAE, IgG(F16)-MMAE and IgG(F6)*-MMAE were analyzed by surface plasmon resonance (BIAcore 3000 System, GE Healthcare) on an TnC-A1-coated CM5 sensor chip (BIAcore) as previously described 15.

Biodistribution studies

The tumor targeting properties of the unconjugated antibodies were assessed by quantitative biodistribution analysis as previously described 37. SIP(F16), IgG(F16) and IgG(F16)* were radioiodinated with ¹²⁵I (PerkinElmer) using Pierce Pre-Coated Iodination Tubes (Life Technologies) and purified on a PD-10 column (GE Healthcare). Immunoreactivity of the labeled proteins were confirmed by affinity chromatography, using the recombinant A1 fragment of Tenascin C coupled to CNBr-activated Sepharose (GE Healthcare) as previously described 37. Balb/c nude mice (Janvier Labs) bearing subcutaneously grafted A431, U87 or MDA-231-MB tumors were injected into the lateral tail vein at a dose of 40 to 80 µg of ADC per mouse. Groups of mice were sacrificed at 24h after injection. Organs were excised, weighed and radioactivity was measured using a Packard Cobra gamma counter. Biodistribution results were expressed as the percentage of injected dose per gram of tissue [%ID/g +/- standard error of the mean (SEM)].

Immunofluorescence studies

Balb/c nude mice bearing subcutaneously grafted A431, U87 or MDA-231-MB tumors were injected with a single dose of 7 mg/Kg of either SIP(F16)-MMAE or IgG(F16)*-MMAE and sacrificed 24 hours after the injection. Tumors were excised, embedded in OCT medium (Thermo Scientific) and cryostat sections (10 μ m) were cut. Slides were stained using the following antibodies: rabbit anti-human IgE (Dako Cytomation), to detect the antibodies in SIP format, rabbit anti-human Fc (Dako Cytomation), to detect the antibodies in IgG format and rat anti-mouse CD31 (BD Biosciences) to detect endothelial cells. Anti-rabbit IgG-AlexaFluor488 (Molecular Probes by Life Technologies) and anti-rat IgG-AlexaFluor594 (Molecular Probes by Life Technologies) were then used as secondary antibodies for microscopic detection of this ex-vivo immuno-staining.

Histo- and Immunohistochemistry

Cut sections of formalin-fixed paraffin-embedded (FFPE) tumors resections were stained by H&E (haematoxylin-eosin) and used for IHC on an automated platform (Ventana Medical Systems). The following antibodies were used: rat monoclonal anti-mouse CD68 (FA11, Serotec Ltd, dilution 1:200), rabbit polyclonal anti-mouse CD31 (Abcam Limited, 1:100) and rabbit polyclonal anti-mouse Myeloperoxidase (Lab Vision Corporation, 1:200). Stained sections were scanned using a high-resolution scanner (Nanozoomer Digital Pathology, Hamamatsu). Histological images were further analyzed with a spatial resolution of 1.84 μ m/pixel. Positive IHC staining (brown color) was detected by color-based thresholding under the L*a*b color space, using a cutoff set by a pathologist (A.S.). IHC scores were computed by dividing number of positive pixels by the corresponding tumor area (manually annotated).

Therapy studies

When tumors reached an average volume of 100-120 mm³ (typically 7 to 9 days after subcutaneous tumor implantation), mice were randomly grouped (n = 3 or n = 5) and injected intravenously (i.v.) into the lateral tail vein.

In the pilot study, mice (n=3) were injected every 3 days, 4 times in total, with the antibody-drug conjugates, either in IgG or in SIP format at a dose of 7mg/Kg, or with vehicle (PBS). In the subsequent therapy study, mice (n=5) were injected every 3 days, 4 times in total, with IgG(F16)*-MMAE at either 10mg/Kg or 3 mg/Kg, or with IgG(KSF)-MMAE at 10mg/Kg, or with free MMAE drug at 0.2 mg/Kg or with vehicle (PBS).

The body weight of mice was monitored daily. Tumor volumes were measured with a digital caliper (volume = length x width² x 0.5). Results were expressed as tumor volume in mm³ +/- SEM. Animals were sacrificed when tumor reached a maximum of 2000 mm³ or weight loss exceeded 15%.

Linker stability assay

ADCs were incubated at a concentration of 150 μ g/mL in mouse serum (Invitrogen) at 37°C in a shaking incubator. At various time point, aliquots were purified by affinity chromatography onto an antigen-coated resin, based on TnC A1 coupled to CNBr-activated

Sephacrose (GE Healthcare), washed with PBS and then eluted with 0.1 M glycine solution (pH = 3), prior to analysis by mass spectrometry.

For *in-vivo* analysis, ADCs were injected in the lateral vein of Balb/c nude mice at a dose of 10mg/Kg. After 24h or 48h, mice were sacrificed and blood was punctured from their heart and transferred into Heparin coated tubes. Tubes were centrifuged at 3000g for 5 min. Plasma was recovered and processed with the same method described above.

Results

Preparation and characterization of ADCs

The F16 antibody was expressed in mammalian cells both in human IgG1 and in small-immune (SIP) format [Figure 1; 27]. In analogy to previous studies [17, 35], the full immunoglobulin format was engineered (through Cys → Ser mutations) to display a single reactive cysteine residue at the C-terminus of the light chain [Figure 1]. In addition, we observed that the Asn-88 residue at the beginning of the CDR3 loop in the VL domain of the antibody was heavily glycosylated in the IgG format [Figure 2], but not in the SIP format. Mutation of the asparagine residue to a glutamine yielded an antibody, IgG(F16)*, with improved antigen binding profiles (as assessed by BIAcore analysis; Supplementary Figure S2) and superior tumor targeting properties (as evidenced in quantitative biodistribution studies, using radiolabeled protein preparations) [Figure 3A/B/C]. A preferential tumor uptake was observed 24h after injection in three models of human tumors (A431, U87 and MDA-MB-231) grafted subcutaneously in nude mice (30, 23 and 17 percent injected dose per gram, respectively). An *ex vivo* microscopic analysis of the antibody localization within the tumor mass revealed a preferential uptake in the antigen-rich sub-endothelial extracellular matrix [Figure 3D].

The F16 antibody mutant, in IgG and SIP formats, was coupled to Vedotin (MC-vc-PAB-MMAE) at a single cysteine residue, yielding homogenous products with drug-antibody ratios of 2:1. Biochemical analysis by SDS-PAGE, size-exclusion chromatography and mass spectrometry confirmed the identity and purity of the products [Figure 1].

Therapy studies

The ADC products in IgG and SIP format, described in the previous section, were first tested in tumor-bearing mice in a pilot experiment [Figure 4A/B]. Nude mice bearing subcutaneously-grafted U87 or A431 tumors received four injections of ADC products at a dose of 7 mg/Kg for both formats (corresponding to an almost double molar amount for the SIP product). The IgG derivative cured 100% of treated mice. The ADC product in SIP format exhibited a substantial anti-tumor activity, which however was not curative [Figure 4A/B]. Encouraged by these initial results, we performed a second therapy study in the A431 model, which confirmed the induction of cancer cures with F16-MMAE at 10 mg/Kg, while a similar ADC product based on the KSF antibody (specific to hen-egg lysozyme and serving as negative control) did not inhibit tumor growth at the same dose [Figure 4C].

We then characterized the stability of the conjugates *in vitro* and *in vivo*, using affinity chromatography on an antigen resin, followed by mass spectrometric analysis. Upon

incubation with mouse serum at 37°C, the SIP-based ADC product exhibited a half-life of approximately 9 hours, while the corresponding IgG derivative was completely stable for three days [Figure 5A]. A similar difference in stability for ADC products based on IgG or SIP antibodies had previously been reported for conjugates featuring disulfide-bonded drugs [17]. It is reasonable to assume that the C-terminal extremity of the SIP antibody may be less sterically hindered, compared to the C-terminal end of the light chain in intact immunoglobulin formats.

Interestingly, IgG(F16)-MMAE was not completely stable *in vivo*, when tested in Balb/c mice. Twenty-four hours after intravenous injection, 75% of the ADC product was intact, while the remaining 25% of the antibody recovered from blood corresponded to the cleavage product at the Citrulline site [Figure 5B].

In order to get more insights about the stability of ADC products and their susceptibility to proteolytic cleavage, we incubated SIP(F16)-MMAE in normal mouse serum or in serum which had been heat-inactivated for 1 hour at 65°C. The conjugate was completely stable in the heat-inactivated sample, indicating that the proteolytic activity could be blocked. This was further confirmed by serum treatment with the irreversible serine protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride [Pefabloc™; Supplementary Table S3]. In addition, diluted solutions of cathepsin B (a cysteine protease which is preferentially active at acidic pH) were able to partially cleave IgG(F16)*-MMAE in mouse serum at pH 6.5, a value which can be found in the tumor extracellular milieu [Supplementary Table S3].

Microscopic analysis

In line with proposed regression scores for human non-small cell carcinoma 39, tumor regression was assessed as percentage of peripheral vital tumor mass per total tumor surface. The A431 cell line derived from a human squamous cell carcinoma entirely recapitulated the microarchitecture of such tumors in our subcutaneous mouse xenograft model: stratification of squamous cell epithelium leads to central keratinization and necrosis. The basal portions directed towards the stroma are most vital and most resistant to chemotherapy, due to best nutrient supply. Response to chemotherapy in squamous cell carcinomas is typically characterized by an expansion of the central necrotic area on cost of the vital rim, which may undergo partial vacuolar change [Figure 6]. Concomitantly, an increase of myeloperoxidase positive intra-tumoral neutrophilic granulocytes was observed [Supplementary Table S4]. Tumor-infiltrating macrophages were rare in our model and not amenable to scoring.

Discussion

We have reported that ADC products, based on antibodies specific to the alternatively-spliced A1 domain of tenascin-C (a non-internalizing antigen), can be potently active against U87 and A431 xenografted tumors. The valine-citrulline linker, used for coupling monomethyl auristatin E to unique cysteine residues in the IgG format, was completely stable in mouse serum but not *in vivo*. The contribution of antibody-based tumor targeting to therapeutic performance was evaluated both by including a negative control antibody in the therapy experiment and by quantitative biodistribution analysis.

The experimental data indicate that peptidic linkers can be cleaved in the tumor extracellular space. Differences in ADC stability were observed when comparing antibodies in IgG and SIP format. Other groups have recently reported striking differences in drug release rate as a result of variations in steric hindrance 40, 41. *In vitro* ADC stability experiments, performed using heat-inactivated mouse serum or pefabloc™ as irreversible protease inhibitor, revealed that serine proteases can substantially contribute to the cleavage of linker-payload [Supplementary Table S3]. These findings are in keeping with the results of Dorywalska et al. 41, who have recently identified carboxylesterase 1C as the main enzyme responsible for the extracellular hydrolysis of valine-citrullin-*p*-aminocarbamate-based linkers in mouse plasma 42. In addition, dilute concentrations of cathepsin B at pH 6.5 can also cleave IgG(F16)*-MMAE [Supplementary Table S3].

ADC products which rely on the extracellular release of cytotoxic payloads may offer distinctive therapeutic advantages 12–14. It has previously been reported that it is difficult for antibodies to reach all cells within a solid tumor mass, as a result of slow diffusion and of “trapping” by antigen on perivascular tumor cells (the so-called “antigen barrier”) 43, 44. Components of the modified tumor extracellular matrix, such as splice isoforms of fibronectin and of tenascin-C, fibrin and certain collagens, are attractive targets for pharmacodelivery applications, because of their selective expression at the tumor site, stability and abundance 18. Drugs released in the tumor extracellular space may diffuse and subsequently internalize into tumor cells, endothelial cells and in tumor-resident leukocytes. Most pharmaceutical developments in the ADC field have so far focused on targets, located on the membrane of tumor cells. In principle internalizing antigens should offer an additional level of selectivity for the development of ADC products. However, in practice, these targets may be suboptimal in terms of *in vivo* pharmacodelivery performance, with the additional limitation that antigen loss by the tumor cells may lead to resistance 45.

In tumor-bearing mice, quantitative biodistribution studies provide unambiguous experimental confirmation of a preferential localization of ADC products at the site of disease 16, 17. In patients, however, very limited knowledge is available, regarding the tumor:organ selectivity that can be obtained with ADCs. Recent advances in antibody radiolabeling and in positron emission tomography should facilitate Nuclear Medicine investigations in patients 3, 46, with considerable benefit for the clinical and industrial development of both internalizing and non-internalizing ADC products. Quantitative biodistribution studies have shown that ligand-based drug delivery approaches may result in a more efficient and selective tumor targeting 16, 17, 47, compared to approaches based on polymer-conjugates which exploit the Enhanced Permeability and Retention (EPR) effect 48–50.

The findings described in this article reinforce the concept that also non-internalizing ADC products may be considered for clinical applications. The novel ADC products, based on the F16 antibody mutant, are potentially suitable for applications in humans, as they incorporate the linker-payload combination of an approved biopharmaceutical (Adcetris™) and as the cognate antigen is strongly expressed in the majority of human malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ADCs	antibody-drug-conjugates
IgG	Immunoglobulin G
MMAE	monomethyl auristatin E
SIP	small immune protein

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Novelty and impact

Antibody-drug conjugates were previously believed to crucially rely on internalization into cancer cells for therapeutic activity. The article shows that non-internalizing antibody-drug conjugates, equipped with peptide-based linker-payload combinations, can efficiently release cytotoxic drugs in the tumor stroma, with potent therapeutic activity in mouse models of human cancer. The study may be of clinical relevance, as it used a clinical-stage antibody (F16) and the linker-payload combination of an approved biopharmaceutical (Brentuximab vedotin).

Figure 1.

Characterization of SIP(F16)-MMAE and IgG(F16)-MMAE. **A**, chemical structure of the MC-vc-PAB-MMAE drug. **B**, schematic representation of SIP(F16)-MMAE and IgG(F16)-MMAE. **C**, SDS-page and size-exclusion chromatography profile of the products. Lanes 1 and 2 represent unmodified antibody in non-reducing and reducing conditions. Please note that, as a result of the Cys-> Ser mutations in the heavy chain, the electrophoretic profile of the two IgG samples is similar. Lane 3 the final MMAE conjugate in non-reducing condition. **D**, ESI-MS characterization of the conjugates. The calculated mass of SIP(F16)-

MMAE and IgG(F16)-MMAE light chain are 39574 and 24020 respectively. (%I = % of MS intensity)

Figure 2. Glycosylation removal on IgG(F16) Light Chain. **A**, SDS-page of IgG(F16) before (lane 1) and after (lane 2) treatment of PNGase F. **B**, ESI-MS spectra of glycosylated IgG(F16) light chain. **C**, ESI-MS spectra of mutated non-glycosylated IgG(F16) light chain.

Figure 3.

Biodistribution and immunofluorescence study of SIP(F16), IgG(F16) and IgG(F16)* in A431, U87 and MDA-MB-231 models. **A/B/C**, Biodistribution study of radioiodinated SIP(F16) (green), IgG(F16) (red) and IgG(F16)* (blue) after a single injection (2 to 4mg/Kg) into balb/c nude mice bearing A431 (**A**), U87 (**B**) or MDA-MB-231 (**C**) tumors. **D**, immunofluorescence analysis performed on sections of A431, U87 and MDA-MB-231 tumors after a single intravenous injection of SIP(F16) (a-c), IgG(F16) (d-f) and IgG(F16)* (g and h, not analyzed in MDA-MB-231 model). The antibody localization on tumor blood vessels was revealed by staining in green (Alexa 488) with anti-human IgE or Fc antibodies whereas the vasculature staining in red (Alexa 594) was provided by anti-CD31 antibodies. Scale bar 50 μ m.

Figure 4.

Therapeutic activity of F16 based ADCs in pilot experiment against A431 epidermal carcinoma (A) or U87 glioblastoma (B) or in main experiment against A431 cells (C). When tumors reached an average of 100 mm³ of volume, mice were randomly grouped and intravenously injected every 3 days, 4 times in total (↑) with the vehicle (○), SIP(F16)MMAE at 7mg/Kg (□), IgG(F16)*-MMAE at 7mg/Kg (A/B) or 10mg/Kg (C) (▲), IgG(KSF)-MMAE at 10mg/Kg (◊) or free MMAE (◇) at 0.2 mg/Kg. Lower

doses of IgG(F16)*-MMAE (e.g., 3mg/Kg) did not induce cures (data not shown). Data points represent mean tumor volume $\pm$ SEM, n=3 (**A/B**) or n=5 (**C**) per group. The right panels show the percentage of body weight change during the course of the therapy.

Figure 5.

A, Kinetics of drug release by ADC incubation in murine serum. The percentage of intact SIP(F16)-MMAE and IgG(F16)*-MMAE products assessed by ESI-MS analysis after incubation in mice serum, and subsequent purification on TnC A1 antigen resin are plotted as a function of time. **B**, MS spectra of IgG(F16)*-MMAE recovered by affinity chromatography after in-vivo incubation in Balb/c nude mice for 24 and 48 hours. Spectra were zoomed in the LC region (22-25 kDa). The peak at 24020 represents the intact LC(F16)-MC-vc-PAB-MMAE while the peak at 23171 represents the LC(F16)-MC-vc,

showing the cleavage after its Citrulline counterpart. (%I = % of MS intensity). Bands were made manually thicker for visual purpose.

Figure 6.

H&E staining of A431 tumor resections at different stage of therapy. Balb/c nude mice were injected with (0, a /1, c /2, d /3, e /4, f) times at 10mg/Kg of IgG(F16)*-MMAE (following the same schedule as for the therapies), and sacrificed 3 days after their last injections. In the left hand panels, black lines highlight the border between central keratinization and necrosis and peripheral vital tumor moiety (scale bar 2.5mm). The percentage of vital tumor mass is displayed in the corner. A descriptive zoom of the panel a is displayed in panel b (scale bar 500 μ m).