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Protease-Cleavable Linkers Modulate the Anticancer Activity of Non-Internalizing Antibody-Drug Conjugates

Alberto Dal Corso^{1,‡}, Samuele Cazzamalli^{1,‡}, Rémy Gébleux¹, Martin Mattarella², and Dario Neri^{1,*}

¹Department of Chemistry and Applied Biosciences, Swiss Federal Institute of Technology (ETH Zürich), Vladimir-Prelog-Weg 4, CH-8093 Zürich (Switzerland) ²Philochem AG, Libernstrasse 3, CH8112 Otelfingen (ZH), Switzerland

Abstract

Antibody-drug conjugates (ADCs) represent an attractive class of biopharmaceutical agents, with the potential to selectively deliver potent cytotoxic agents to tumors. It is generally assumed that ADC products should preferably bind and internalize into cancer cells in order to liberate their toxic payload, but a growing body of evidence indicates that also ADCs based on non-internalizing antibodies may be potently active. In this Communication, we investigated dipeptide-based linkers (frequently used for internalizing ADC products) in the context of the non-internalizing F16 antibody, specific to a splice isoform of tenascin-C. Using monomethyl auristatin E (MMAE) as potent cytotoxic drug, we observed that a single amino acid substitution of the Val-Cit dipeptide linker can substantially modulate the *in vivo* stability of the corresponding ADC products, as well as the anticancer activity in mice bearing the human epidermoid A431 carcinoma. In these settings, the linker based on the Val-Ala dipeptide exhibited better performances, compared to Val-Cit, Val-Lys and Val-Arg analogues. Mass spectrometric analysis revealed that the four linkers displayed not only different stability *in vivo*, but also differences in cleavage sites. Moreover, the absence of anticancer activity for a F16-MMAE conjugate featuring a non-cleavable linker indicated that drug release modalities, based on proteolytic degradation of the immunoglobulin moiety, cannot be exploited with non-internalizing antibodies. ADC products based on the non-internalizing F16 antibody may be useful for the treatment of several human malignancies, as the cognate antigen is abundantly expressed in the extracellular matrix of several tumors, while being virtually undetectable in most normal adult tissues.

†Author Contributions

A.D.C. and S.C. contributed equally to this work.

*Corresponding Author: neri@pharma.ethz.ch; Tel: +41-446337.

ORCID

Alberto Dal Corso: 0000-0003-4437-8307

Samuele Cazzamalli: 0000-0003-0510-5664

Notes

Dario Neri is co-founder, shareholder and Board Member of Philogen, the company that owns the F16 antibody.

Introduction

The use of cytotoxic agents represents the backbone of many pharmacologic approaches for the treatment of cancer. Conventional chemotherapy is often limited by the inefficient accumulation of small anti-cancer drugs into solid tumors, as evidenced by quantitative biodistribution studies and by Nuclear Medicine studies in cancer patients using radiolabeled drugs.^{1,2} Suboptimal pharmacokinetic profiles may damage normal organs and prevent dose escalation of chemotherapeutic agents to therapeutically active regimens. The conjugation of cytotoxic compounds to monoclonal antibodies, capable of selective accumulation at the tumor site, yields a class of promising biopharmaceutical agents, termed “antibody-drug conjugates (ADCs)”. In most cases, antibodies capable of selective binding and subsequent internalization into tumor cells are used, with the aim to release cytotoxic payloads inside the tumor site, thus improving therapeutic index.^{3,4,5,6} Two ADC products are available on the market for different indications (i.e., brentuximab vedotin for the treatment of certain hematological malignancies and ado-trastuzumab emtansine for the second-line treatment of HER2-positive metastatic breast cancer), while over 50 products are now under clinical evaluation.⁷

Brentuximab vedotin contains a linker-payload combination, featuring a valine-citrulline (Val-Cit) dipeptide moiety, a self-immolative spacer and monomethyl auristatin E (MMAE) as toxic drug.⁸ The Val-Cit linker was chosen and optimized, in order to facilitate an intracellular cleavage by lysosomal cysteine protease (mainly cathepsin B) and the following release of MMAE, upon elimination of the *p*-aminobenzyl self-immolative spacer. It has recently been shown, however, that derivatives certain plasma enzymes, such as carboxylesterase 1C, can partially digest the Val-Cit linker in some ADC products, leading to drug release before the internalization process.⁹

Although it has been generally assumed that antibody internalization represents a strict requirement for the ADC efficacy,^{10,11} recent studies indicate that non-internalizing ADCs may also lead to efficacious products. Evidence of potent preclinical activity in models of cancer has been reported for non-internalizing ADC products, directed against a number of targets, including collagen IV¹² and fibrin¹³ as well as splice variants of fibronectin^{14,15} and tenascin-C.¹⁶

The clinical-stage F16 antibody, specific to the alternatively-spliced A1 domain of tenascin C, does not internalize into tumor cells, but stains extracellular matrix structures in the majority of solid tumors and haematological malignancies, but not in normal tissues.¹⁷ The ability of the F16 antibody to preferentially localize to tumors has been demonstrated using radiolabeled preparations, both in preclinical models¹⁷ and in cancer patients.¹⁸ When the F16 antibody in IgG format was functionalized at a single cysteine site with a Val-Cit-MMAE linker-payload combination, the resulting non-internalizing ADC product exhibited a potent and selective anti-cancer activity in mice bearing A431 tumors and U87 glioblastomas.¹⁹

In principle, many proteolytic enzymes within the tumor microenvironment may influence the activity and selectivity of non-internalizing ADC products, featuring peptide-based

linkers. In this paper, we show that the use of cleavable linkers in the IgG(F16)-MMAE conjugates represents a strict requirement for *in vivo* therapeutic activity. In particular, the dipeptides Val-Ala and Val-Cit exhibited a better performance compared to Val-Lys and Val-Arg for the antibody-based delivery and release of MMAE, in tumors rich in splice variants of tenascin-C.

Results and Discussion

According to literature data, linkers featuring the Arg residue at the C-termini show high cleavage rates in the presence of different cysteine cathepsins, abundantly expressed at the tumor site.²⁰ Historically, the Arg moiety has been either protected or replaced with lysine and citrulline residues, in order to improve the systemic stability.²¹ While this research activity led to the selection of Val-Cit dipeptide as optimal linker for internalizing ADCs, we investigated the use of Val-Cit, Val-Arg and Val-Lys sequences, as linkers for the delivery of MMAE from non-internalizing ADCs. Finally, the Val-Ala dipeptide was also studied, being used as linker in both ADCs²² and small molecule-drug conjugates.^{23,24} These four dipeptides were functionalized with the *p*-aminobenzyl carbamate (PABC) self-immolative spacer and the MMAE payload at their C-termini, according to published procedures (Figure 1).²⁵ ADC products were prepared using an engineered derivative of the F16 antibody in human IgG1 format, bearing a reactive cysteine residue at the C-terminus of the light chain, while the other cysteine residues in the hinge region of the heavy chain had been mutated to serine.¹⁹

The strategy yielded ADCs with defined drug/antibody ratio (DAR) of 2, which was confirmed by mass spectrometry. Product homogeneity was also confirmed by SDS-PAGE analysis and analytical size-exclusion chromatography (Figure 2).

A dose escalation experiment was used to determine the maximum tolerated dose (MTD) of the newly prepared F16-MMAE ADCs. Healthy BALB/c nude mice were treated with compounds **1-4**, administered intravenously with three cycles of injections, at 12.5, 25 and 50 mg/kg doses. Daily analysis of body weights indicated that all ADC products were well tolerated, even at the highest administered dose (Figure 3A).

The anticancer performance of ADCs **1-4** was investigated in a first therapy experiment in BALB/c nude mice, subcutaneously grafted with human epidermoid carcinoma cells (A431). When tumors reached 100 mm³ volume, mice were randomized and four intravenous administrations of F16-MMAE conjugates were performed following the schedule indicated in Figure 3B. While the reference ADC product F16-Val-Cit-MMAE (**1**) had previously shown curative effects when administered in four cycles at the dose of 7 mg/kg,¹⁹ we treated mice at a dose of 3 mg/kg, in order to identify the linker structure associated with the strongest anticancer effect at a suboptimal dose. All F16-MMAE conjugates inhibited tumor growth compared to the saline control (Figure 3B). F16-Val-Arg-MMAE (**2**) showed the weakest activity, whereas F16-Val-Ala-MMAE (**4**) exhibited the best tumor growth inhibition.

In order to formally prove that a cleavable peptide moiety was essential for activity, we prepared a fifth F16-MMAE conjugate, structurally similar to the previous conjugates but featuring a non-peptidic amide bond instead of a cleavable dipeptide. Similar non-cleavable (NC) linkers have often been used to couple different classes of cytotoxics (e.g. maytansinoids,²⁶ calicheamicins,²⁷ cryptophycins²⁸ and auristatins²⁹) to internalizing antibodies, with the aim of selectively release the toxic moiety in intracellular compartments, exploiting a proteolytic degradation of the immunoglobulin. The non-cleavable linker-MMAE module was thus synthesized by direct coupling of 6-maleimidoheptanoic acid (compound **5** in Figure 4) to 4-aminobenzyl alcohol, which was then converted into the 4-nitrophenyl carbonate **6** and reacted with MMAE. The resulting structure was purified by HPLC and conjugated to the F16 mAb, leading to the new ADC F16-NC-MMAE (**8**, Figure 4B,C).

All F16-MMAE conjugates were then subjected to a comparative analysis of *in vivo* linker stability and metabolism. ADC products **1-4** and **8** were intravenously injected into tumor-bearing mice and animals were sacrificed after 24 and 48 hours. Blood samples were collected by heart puncture, followed by plasma purification and ADC extraction through filtration over antigen-coated resin. MS analysis of the F16 light chain revealed the progressive formation of different linker fragments, covalently bound to the protein. The metabolite occurrence was quantified by comparing the intensity of the MS signal relative to the light chain bearing the truncated fragment to the one of the intact conjugate. The results of this analysis are shown in Figure 5, together with a schematic representation of the observed metabolites.

With the exception of the maleimide hydrolysis (see “fragment LC-Mc(H₂O)-linker-MMAE” in Figure 5) all the observed fragments are consistent with the release of MMAE payload from the antibody vehicle. As expected, the rate of MMAE loss from F16-Val-Arg-MMAE ADC was the largest one (compared to the other three dipeptide linkers), whereas the non-cleavable analogue showed an excellent *in vivo* stability for 48 hours.

An interesting feature was observed in ADCs bearing a basic side chain in the linker (i.e., ADCs **2** and **3**) and not in the Val-Cit and Val-Ala counterparts. While the linker in the latter two ADCs was only cleaved at the C-terminal position, the presence of basic residues in the other dipeptides was associated to the formation of a different main metabolite, consistent with the cleavage at the Valine C-terminus (i.e., fragment LC-Mc-Val-OH, reported as compound **14** in Scheme 1).

During the analysis of F16-Val-Lys-MMAE, both fragments LC-Mc-Val-OH (**14**) and, to a lesser extent, LC-Mc-Linker-OH (**13**) were detected. We interpreted the data as a result of Lysine digestion after the “traditional” dipeptide cleavage (i.e., *Cut 1* followed by *Cut 2* in Scheme 1). By contrast, metabolism of F16-Val-Arg-MMAE only led to the formation of fragment LC-Mc-Val-OH (**14**). This data may indicate that the arginine proteolysis (*Cut 2*) was too fast to allow the detection of intermediate **13**, even though a second pathway consisting in the direct cleavage of the dipeptide at the internal amide bond (*Cut 2'*) may also occur. The inclusion of ADC F16-NC-MMAE **8** in the experiment allowed the evaluation of metabolic processing in living animals of all the linker components (e.g., the

maleimide moiety and the carbamate bond between the PAB spacer and MMAE) in the absence of dipeptide moieties. In particular, the maleimide hydrolysis is known to inhibit the retro-Michael step that results in the complete deconjugation of the linker-payload module (i.e., fragment LC-SH).³⁰ The free F16 antibody was detected in blood either in the reduced form (e.g., free thiol) or oxidized by free cysteine present in circulation (i.e., fragment LC-SS-Cys), as already reported in the literature.³¹

Overall, these data indicated that the ADC F16-Val-Cit-MMAE showed similar metabolic profiles and rate of MMAE loss to its analogue featuring the Val-Ala linker. The latter has been used as linker in internalizing ADC products, featuring pyrrolbenzodiazepine (PBD) as cytotoxic payload.²² One of these therapeutics, Vadastuximab Talirine (SGN-CD33A), has recently entered Phase III trials with the CASCADE study in patients with newly diagnosed acute myeloid leukemia (AML), in combination with azacitidine or decitabine. F16-MMAE ADC based on Val-Cit or Val-Ala were highly active, when used at the dose of 7 mg/kg (at which similar conjugates based on irrelevant negative control antibodies do not display detectable anti-cancer activity).¹⁹ By contrast, tumors grew rapidly in animals treated with vehicle (PBS) or with the non-cleavable F16-NC-MMAE product **8** (Figure 6).

These findings are in keeping with the metabolite analysis described above, confirming that the Mc-NC-MMAE linker is stable both in circulation and in the extracellular tumor environment. Very recently, Mendelsohn and coworkers have reported that the internalizing mAb trastuzumab, functionalized with carbamate-based non-cleavable linkers, do not show anticancer activity *in vitro* or *in vivo*.³²

It is likely that drug release processes and rates in the extracellular space are similar for internalizing and non-internalizing ADCs. An experimental characterization of the portion of ADC product that is internalized into tumor cells (and of the subsequent drug release kinetics) is extremely difficult to perform *in vivo*, especially in patients. In the context of non-internalizing ADCs, our group observed and reported an interesting correlation between the linker system and the antibody format: while ADCs bearing disulfide linkers have shown superior anticancer properties when the mAb is expressed as small immune protein (SIP),³³ drug release in the extracellular space upon proteolytic action was found to be more efficient when IgG1-based mAbs are used.¹⁹

Extracellular matrix components are abundant and easily accessible in perivascular areas of tumors, which can significantly improve the accumulation and persistence of macromolecular therapeutics at the site of disease.³⁴ Payloads released in the extracellular environment may diffuse within the tumor mass, thus potentially reaching a large number of cancer cells³⁵, into which they would eventually internalize. The induction of tumor cell death may lead to a release of proteolytic enzymes which, in turn, could trigger additional drug release from a non-internalizing ADC product, in process which is reminiscent of a “chain reaction”. It remains to be seen, however, to which extent preclinical findings observed in tumor-bearing mice may be predictive for the protease-driven activation of ADCs in human malignancies, as enzyme concentrations could be different in the two species.

Conclusion

We have shown that ADC products, based on non-internalizing antibodies and featuring dipeptide cleavable linkers, can be potently active against cancer *in vivo*, while similar products based on non-cleavable linkers are not active. The linker-payload combination Val-Cit-MMAE, successfully used for the internalizing Adcetris™ ADC product, holds promise also in the context of non-internalizing tumor-targeting antibodies.

Conjugates between the IgG(F16) antibody and the potent MMAE auristatin may be considered for the treatment of various conditions, since the A1-extra domain of tenascin C is abundantly expressed in the extracellular matrix of various tumor lesions (including non-small cell lung cancer,³⁶ head and neck³⁷ and breast³⁸ cancers, melanoma³⁹, as well as certain lymphomas⁴⁰ and leukemias,⁴¹ while being undetectable in most normal adult tissues.

Supporting Information

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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Figure 2.

Synthesis and characterization of F16-MMAE ADCs **1-4**. **A**) Schematic representation of the conjugation protocol. TCEP: tris(2-carboxyethyl)phosphine. **B**) size-exclusion chromatography and **(C)** SDS-page profiles of the products in either reducing (R) and non-reducing (NR) conditions; **D**) deconvoluted MS spectra: antibody light chains (LC) are functionalized with Mc-Linker-MMAE modules, while the heavy chain (HC) is glycosylated, resulting in 3 main peaks of ca. 50 KDa mass (calcd. HC mass: 48489.58). Calcd. mass of the light chains of ADCs **1-4** are 24023.90, 24022.92, 23994.90 and

23937.81, respectively (4 Cys residues are considered reduced in the calculation; calcd. mass of non-conjugated LC: 22707.25). Attribution of common extra-peaks of MS spectra is indicated in ADC **1** spectrum (deconvolution artifacts *: LC/2; **: HC/4; ***: HC/3; ****: HC/2, *****: LC·2; *u*: trace of non-functionalized LC). Full MS spectra (raw and deconvoluted, including non-conjugated F16 antibody) are shown in the Supporting Information.

Figure 3.

Biological evaluation of F16-MMAE conjugates **1-4**, featuring different cleavable dipeptide linkers: **A**) dose escalation study (BALB/c nude mice, $n = 1$ per group); **B**) Therapeutic activity of F16-MMAE ADCs against A431 human epidermoid carcinoma xenografted in BALB/c nude mice, after 4 administrations of ADCs (3 mg/kg every 3 days, as indicated by the arrows) and vehicle (PBS). A graph showing the percentage of body weight changes during the experiment is included in the Supporting Information. Data points represent mean tumor volume $\pm$ SEM, $n = 4$ per group. ** indicates $p < 0.01$; * indicates $p < 0.05$.

Figure 4.

A) Synthesis of the linker-drug module Mc-NC-MMAE (**7**). Reagents and Conditions: a) [1] 4-aminobenzyl alcohol, EDC·HCl, *i*Pr₂Net, CH₂Cl₂, r.t. overnight; [2] 4-nitrophenyl chloroformate, pyridine, THF, r.t. 4 h; b) MMAE·TFA, HOAt, *i*Pr₂Net, DMF, r.t. 48 h. **B)** size-exclusion chromatography and **(C)** SDS-page profiles of the F16-NC-MMAE ADC (**8**) in either reducing (R) and non-reducing (NR) conditions.

Figure 5.

Schematic representation of the linker fragments observed by MS spectroscopy and presentation of their relative abundance after 24 and 48 h post injections in tumor-bearing mice ($n = 2$ mice/ADC). Data correspond to the ratio between the MS peak intensity of individual fragments and the sum of MS signal intensities of all detected peaks (100%). MS data of ADCs **2**, **4** and **8** are shown in Figure S1. LC = light chain of F16 mAb.

Figure 6.

A) Therapeutic activity of F16-Val-Cit-MMAE (**1**), F16-Val-Ala-MMAE (**4**) and F16-NC-MMAE (**8**) against A431 human epidermoid carcinoma xenografted in BALB/c nude mice, after 4 administrations of ADCs (7 mg/kg every 3 days, as indicated by the arrows) and vehicle (PBS); **B)** body weight changes (%) of the different treatment groups during the therapy experiment. Data points represent mean tumor volume $\pm$ SEM, $n = 5$ per group.

Scheme 1.

Proposed proteolytic pathways of ADCs **2** and **3**, bearing amino acids with basic side chains in the linker module.