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Optimization of Adcitmer, a Monomethyl-Auristatin E bearing antibody-drug conjugate for the treatment of CD56-expressing cancers

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ABSTRACT

The cell adhesion protein CD56 has been identified as a potential therapeutic target in several solid tumors and hematological malignancies. Recently, we developed a CD56-directed antibody-drug conjugate (ADC), called Adcitmer and demonstrated its antitumor properties in preclinical models of the rare and aggressive skin cancer Merkel cell carcinoma (MCC).

The present study aims to further optimize Adcitmer to overcome the therapeutic limitations observed with previously evaluated CD56-targeting ADCs, which were partially related to toxic effects on leukocytes. To this end, we aimed to avoid interaction of Adcitmer with immune cells via Fc gamma receptor (FcγR) binding. Since glycosylation is essential for FcγR binding, an aglycosylated form of Adcitmer was generated and evaluated on human leukocytes and MCC cell lines using cell death (annexin V/*7-aminoactinomycine D*) and proliferation (2,3-Bis-(2-methoxy-4Nitro-5-sulfophenyl)-2H-tetrazolium-5carboxanilide) assays. Finally, the therapeutic performance of Adcitmer and its aglycosylated form was assessed in an MCC xenograft mouse model. Investigating the Adcitmer interaction with immune cells demonstrated that it is mostly mediated by Fc recognition. Accordingly, Adcitmer aglycosylation led to reduced immune cell toxicity and strikingly also to improved therapeutic performance even in an MCC xenograft model using immunodeficient mice.

Our study suggests that aglycosylated Adcitmer should be considered as a therapeutic option in patients with advanced MCC or other CD56-positive tumors.

INTRODUCTION

Antibody-drug conjugates (ADCs) development constitutes a therapeutic field in expansion, with more than hundreds of ADCs in preclinical and clinical development and 11 molecules already approved by the Food and Drug Administration in the field of oncology.[1]

ADCs consist of a monoclonal antibody and a highly potent cytotoxic agent (ie, payload), associated by a linker.[2 3] The performances of these drugs rely on antibody properties,[4–6] on the nature of the cytotoxic drug, but also on the bioconjugation technology.[1 7] Another determinant of ADC efficiency is the bystander effect, that is, the ability of the ADC to induce cell death through the release of the cytotoxic molecule into the tumor microenvironment.[8–11]

Besides the targets of the current approved ADC, several other membrane antigens have been identified over the last few years as potentially relevant for ADC-based strategy. Among them, CD56, also known as neural cell adhesion molecule, is expressed in various neuroendocrine neoplasms including small cell lung cancer (SCLC) and Merkel cell carcinoma (MCC).[12] Notably, a first-generation CD56-targeting ADC named lorvotuzumab mertansine (LM) has previously been generated and evaluated in phase I/II trials.[13–15] While acceptable tolerability and signals of efficacy, especially in patients with MCC, have been observed when used in monotherapy, severe neutropenia and sepsis were evidenced when combined with chemotherapy in patients with SCLC.[14]

Recently, using an optimized bioconjugation technology,[16 17] our group generated and evaluated a new CD56-targeting ADC (PCT/FR2021/050332) called Adcitmer for treatment of MCC, a rare and aggressive skin cancer. This ADC was generated from the bioconjugation of the CD56-targeting human monoclonal antibody m906 to a cytotoxic drug (monomethyl auristatin E (MMAE),

currently the most used payload in clinic[1 18]) using the McSAF Inside bioconjugation process.[19] Using MCC xenograft on immunodeficient mice, Esnault *et al* demonstrated the ability of Adcitmer to reduce tumor growth compared with non-binding control ADC.[19]

In this context, the present study aims to further optimize Adcitmer to overcome a therapeutic limitation—namely hematologic toxicity—observed with previously tested CD56-targeting ADCs.

MATERIALS AND METHODS

Cell lines

MCC cell lines WaGa[20] (RRID: CVCL_E998), PeTa[21] (RRID: CVCL_LC73), MS-1[20] (RRID: CVCL_E995), MKL-1[22] (RRID: CVCL_2600) and MKL-2[23] (RRID:CVCL_D027) were grown in RPMI (Roswell Park Memorial Institute) medium containing 10% fetal calf serum, 1% penicillin, streptomycin and amphotericin B. WaGa, PeTa and MKL-1 CD56 knockout (KO) have been generated as previously described.[19]

ADCs binding to Fc gamma receptor

Adcitmer, aglycosylated Adcitmer and LM binding to CD16 and CD64 were evaluated using an ELISA competition assay following the manufacturer's instructions (Lumit FcγR Binding Immunoassay, Promega).[24] Detection was performed on Luminoskan Ascent (Thermo Fisher).

Evaluation of toxicity on neutrophils, natural killer cells and monocytes

All primary cells were isolated from PBMCs (Human peripheral blood mononuclear cells) of healthy donors (online supplemental material and methods). Natural killer (NK) cells and monocytes were incubated in culture medium (online supplemental material and methods) in the presence of different doses (from 20 to 900 nM) of Adcitmer, aglycosylated Adcitmer, m906, aglycosylated m906 and LM at 37°C, 5% CO_2. Cell death induced by the different treatments was analyzed by APC (Allophycocyanin)-annexin V and 7-AAD (*7-aminoactinomycine D*) staining[20] (APC annexin V Apoptosis Detection Kit with 7-AAD, BioLegend), and was measured by flow cytometry (CytoFLEX, Beckman Coulter). Treatment durations were 4 hours and 4 days for NK cells and only 4 hours for monocytes and neutrophils since these cells did not express the CD56 and only ADCC (antibody-dependent cell cytotoxicity) phenomenon is evaluated. Percentages of cell death were calculated by counting the 7-AAD/annexin V positive cells in each condition compared with the untreated cells.

Proliferation assay

To evaluate cell viability and metabolic activity, XTT ((2, 3-Bis-(2-methoxy-4Nitro-5-sulfophenyl)-2H-tetrazolium-5 carboxanilide) assays were performed according to standard protocols.[19] Cells were plated in three replicates in a 96-well plate with 5×10^4 cells/well. Adcitmer, aglycosylated Adcitmer, LM and MMAE were added in equivalent incremental concentrations, considering that each antibody bears four MMAE molecules. Untreated cells were used as viability references, while cells treated with 1% Triton X-100 served as positive controls for cell death. After 4 days, 25 µL XTT reagent at 1 mg/mL (Alfa Aesar, Thermo Fisher) with N-methyl dibenzopyrazine methyl sulfate activator (25 µM) was added per well, then absorbance was measured at 450 nm after 4 hours incubation, and absorbance at 620 nm was used as a reference. Three independent experiments were performed.

Merkel cell carcinoma tumor mice model

All animal procedures were approved by the local ethics committee (N° ID RCB 2009-A01056-51; Apafis# 40588–2023013014069196-v3). Twenty-six 7-week-old females NOD/SCID mice (Janvier Labs) were maintained and fed under aseptic conditions. Isoflurane-anesthetized mice received one subcutaneous injection of 10^7 WaGa cells with 10% matrigel on the right flank[19 25]. The general state of animals and body weight was monitored twice a week during the entire procedure. Tumor volume was measured with a caliper, and tumor volume was obtained using $\pi/6\times$ width × length × height. When tumor volume reached 150 mm^3, mice were included in the study and randomly assigned in double-blinded fashion to the different groups. The inclusion period was limited to 2 weeks to prevent heterogeneous growth tumor inclusion. After inclusion, animals (n=6/group) received twice a week intravenous injection of ADC at 5 mg/kg (either Adcitmer or aglycosylated Adcitmer) or volume-equivalent injection of phosphate-buffered saline (PBS) with a total of nine injections. Mice were sacrificed 30 days after inclusion or when mice reached the predefined endpoint (tumor ulceration, weight loss >20%, or prostration). Tumor, heart, lungs, spleen, and liver were removed for macroscopic examination. After dissection, tumor weight and volume were assessed. To detect metastatic spread and potential toxicity of ADCs, whole tumors, and organs (heart, lungs, spleen, and liver), were formalin-fixed and paraffin-embedded for microscopic evaluation to evaluate architecture changes, inflammatory infiltrate, necrosis, vascular changes, fibrosis, and liver steatosis.

Statistical analysis

Data are described as mean±SEM, or median±quartile. Associations were assessed by Mann-Whitney or two-way analysis of variance tests for continuous data. To determine correlation, the Pearson's test was performed. For all statistical analyses, GraphPad Prism (GraphPad, La Jolla, California, USA) was used. *p<0.05, **p<0.005, ***p<0.0005 were statistically significant.

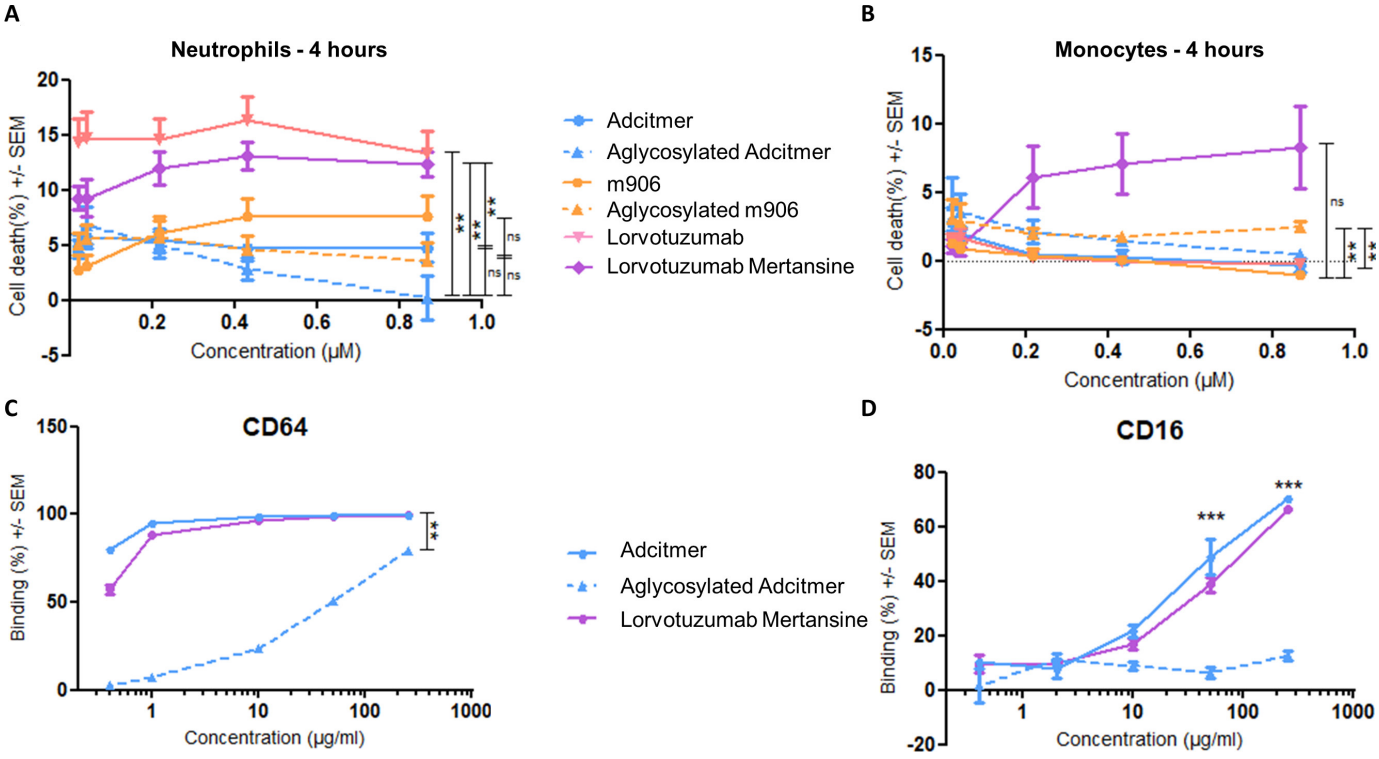


Figure 1 Aglycosylation of Adcitmer impairs FcγR binding and reduces off-target effects on immune cells. Cell death of neutrophils (A) and monocytes (B) was analyzed by annexin V/7-AAD staining after 4 hours of incubation with the indicated substances at 37°C with a range of concentration. Experiments were performed three or four times independently. Data are expressed as mean frequency of dead cells±SEM. Data are expressed as mean percentage of binding±SEM of (C) FcγRIa or CD64 and (D) FcγRIIIa or CD16. The binding was evaluated using an ELISA kit with Adcitmer, aglycosylated Adcitmer or lorvotuzumab mertansine in a concentration range after 6 min of substrate incubation times. 7AAD, *7-aminoactinomycine D*; FcR, Fc gamma receptor.

RESULTS

Cytotoxicity of Adcitmer and LM on myeloid cells

As severe hematologic toxicities including neutropenia were observed in patients treated with LM, we first aimed to compare the potential cytotoxic effect of Adcitmer and LM in vitro on neutrophils and monocytes, two immune populations lacking CD56 expression[26 27].

Naked antibodies and ADC were incubated with monocytes and neutrophils for 4 hours. At that time, LM was cytotoxic on both monocytes and neutrophils (mean percentage of mortality=10% ±2.9 and 13% ±1.8, respectively, at 0.9 µM (highest tested dose)) while Adcitmer exerted lower killing effect than LM on neutrophils (mean percentage of mortality=5% ±1.3 at the same dose, p=0.0079; figure 1A) and had no significant effect on monocytes (figure 1B). Interestingly, similar results were observed with the m906 and lorvotuzumab antibodies alone (figure 1A,B).

Aglycosylation of Adcitmer prevents interaction with immune cells

To test whether such an effect might be due to the recognition of the Fc portion of the ADCs to the Fc gamma receptor (FcγR) on immune cells, we then generated an aglycosylated version of Adcitmer by introducing a point mutation at asparagine 297 to alanine (N297A), which had been described[28 29] to largely reduce Fc-FcγR interaction. Following this antibody engineering, we confirmed the decrease of binding to FcγRIIIa and FcγRIa (figure 1C,D) and absence of toxicities of the aglycosylated antibody and ADC on myeloid cells (figure 1A,B). These findings confirm that the binding of LM, and to a lesser extent Adcitmer, to myeloid cells is associated with toxicities in these populations.

We then evaluated the effect of LM, Adcitmer and aglycosylated Adcitmer on NK cells, an immune population expressing CD56 (figure 2A). After 4 hours of incubation, a significant killing effect was observed on NK cells for both LM and Adcitmer with higher cell mortality in cells treated with LM (mean percentage of mortality=33% ±1.8 vs 7.4% ±0.8 at 0.9 µM (highest tested dose); p=0.008; figure 2B). Similar findings were observed after 4 days of incubation with a mean percentage of mortality of 65% ±12 for LM and 36% ±8.9 of mortality for Adcitmer (p=0.008; figure 2C). Importantly, aglycosylation of Adcitmer resulted in a drastic decrease in mortality (mean percentage of mortality=1.7% ±1.4 at 0.9 µM after 4 hours and 16% ±5 after 4 days; figure 2B,C) suggesting that toxicities on NK cells observed with the glycosylated ADCs are dependent on the Fc part recognition. Since the unconjugated CD56 targeting antibody m906 but not its aglycosylated form mediated cytotoxicity on NK cells after 4 hours

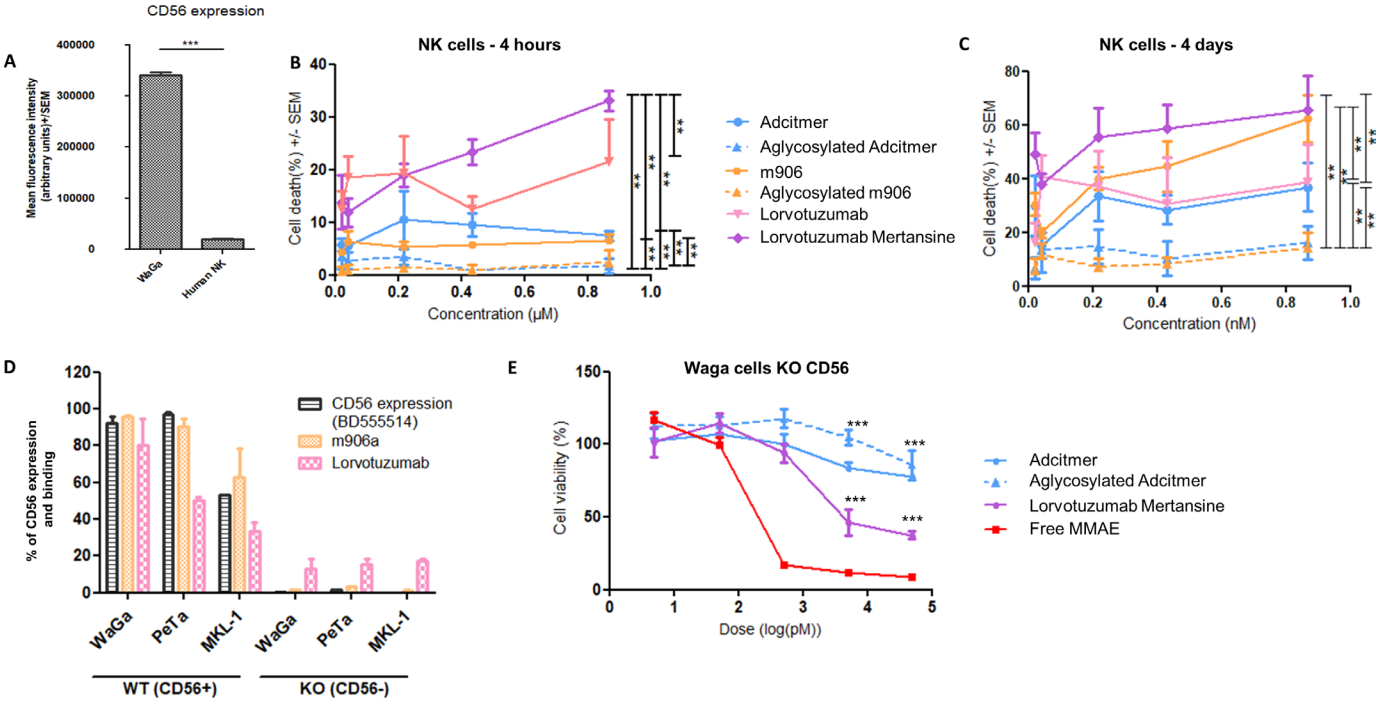


Figure 2 Aglycosylation of Adcitmer reduces off-target effect on NK cells. (A) Evaluation of CD56 expression in WaGa and NK cells by flow cytometry. Data are expressed as mean fluorescence intensity. Cell death of NK cells was assessed by annexin V/7-AAD staining after 4 hours (B) or 4 days (C) of incubation with the indicated substances at 37°C. (D) Results are presented as percentages mean of binding±SEM after incubation with aglycosylated m906 and lorvotuzumab. (E) Viability of the CD56 knockout cells (WaGa CD56 KO) was assessed by XTT assay. Untreated cells were used as a reference. Experiments were conducted as triplicates and were performed three times independently. Data are presented as mean percentage of dead or viable cells±SEM. 7AAD, *7-aminoactinomycine D*; KO, knockout; MMAE, monomethyl auristatin E; NK; natural killer; WT, wildtype; XTT, ((2,3-Bis-(2-methoxy-4Nitro-5-sulfophenyl)-2H-tetrazolium-5carboxanilide).

(figure 2B), this suggests that at least part of the cytotoxic effect of Adcitmer on NK is due to ADCC.

Altogether, these results suggest that LM and, to a lesser extent, Adcitmer exert toxicities on immune cells through several mechanisms, including Fc recognition, and that aglycosylation of Adcitmer can dramatically reduce these effects.

Evaluation of the cytotoxic effect of aglycosylated Adcitmer on Merkel cell carcinoma cell lines in vitro

To evaluate whether aglycosylation of Adcitmer might impact its antitumor properties, we then compared the biological properties of Adcitmer, aglycosylated Adcitmer and LM on MCC cell lines in vitro.

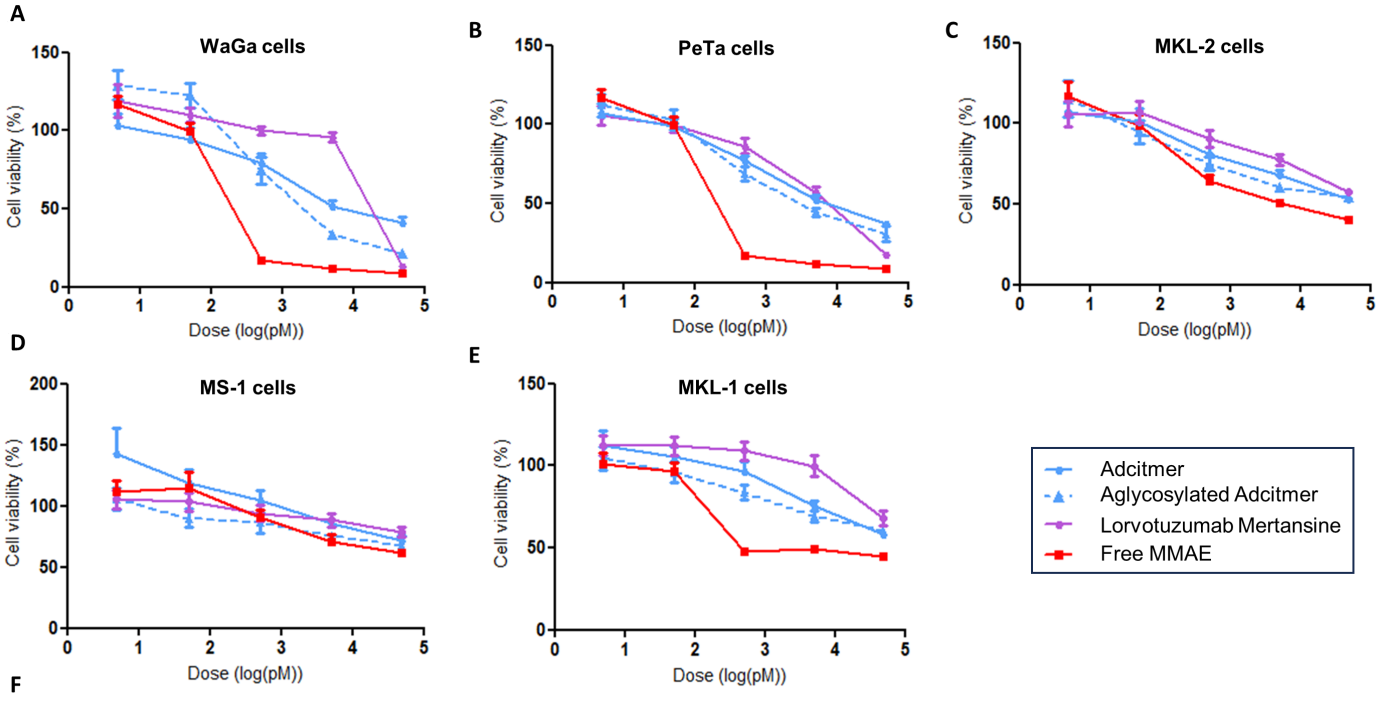
First, the binding of the unconjugated antibodies (aglycosylated m906 and lorvotuzumab) to three MCC cell lines either expressing CD56 or CRISPR/Cas9 CD56 KO cell lines[19] was evaluated by flow cytometry. Interestingly, a signal was obtained with lorvotuzumab; no binding was observed for aglycosylated m906 on the CD56 KO cells (figure 2D).

ADC and free MMAE cytotoxicity was then assessed in vitro on CD56 KO WaGa cells (figure 2E) and on MCC (figure 3A–E). A significant cytotoxic effect was observed after exposure of WaGa CD56 KO cells to LM (inhibitory concentration 50% (IC50)=6.8 nM), while Adcitmer and its aglycosylated version did not impair cell viability (figure 2E). IC_{50} values were lower for aglycosylated compared with Adcitmer and 1.9-fold to 7.6-fold lower than for LM (figure 3). To note, significant differences in sensitivity to Adcitmer or its aglycosylated form were found according to the MCC cell line tested, but no correlation was found between the IC_{50} calculated and their CD56 expression (online supplemental figure 1A,B). In fact, sensitivity to Adcitmer rather seemed to depend on MMAE sensitivity of these cell lines (figure 3).

These results demonstrate that the aglycosylation of Adcitmer does not reduce its cytotoxic properties on MCC cell lines in vitro and further suggest that binding of LM, independently of CD56 expression, might result in unspecific toxicities.

Aglycosylation of adcitmer enhances antitumor growth effect in Merkel cell carcinoma xenograft mice model

Finally, we evaluated the antitumor performance of aglycosylated Adcitmer in vivo in a previously described MCC tumor xenograft mice model.[19] Briefly, WaGa cells were subcutaneously injected in NOD-SCID mice. After the development of 150 mm^3 tumors, mice received intravenous injection of Adcitmer, aglycosylated Adcitmer (5 mg/kg) or PBS twice a week for 1 month (total nine injections). In contrast to our initial experiments,[19] a short period of prostration (5 min duration) was observed after the first injection in all animals treated with Adcitmer.



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IC50 (nM)	WaGa	PeTa	MS-1	MKL-1	MKL-2
Aglycosylated Adcitmer	2.55	3.75	85.67	55.94	33.65
Adcitmer	7.79	7.53	116.67	57.71	39.38
LM	18.99	6.77	169.63	111.59	55.81
MMAE	0.77	0.28	61.20	3.24	5.85

Figure 3 Aglycosylated Adcitmer cytotoxicity against Merkel cell carcinoma cell lines. After 4 days of exposure to Adcitmer, aglycosylated Adcitmer, LM or free MMAE, viability of the (A) WaGa cells. (B) PeTa cells. (C) MKL-2 cells. (D) MS-1 cells or (E) MKL-1 cells was assessed by XTT assay. Untreated cells were used as a reference. Experiments were conducted as triplicates and were performed three times independently. Data are expressed as mean percentage of viable cells±SEM. (F) The half-maximal inhibitory concentration (IC50) value was determined using GraphPad Prism. LM, lorvotuzumab mertansine; MMAE, monomethyl auristatin E; XTT, ((2,3-Bis-(2-methoxy-4Nitro-5-sulfophenyl)-2H-tetrazolium-5carboxanilide).

The symptomatology was characterized by piloerection and reduced activity. No symptoms were observed in animals treated with the aglycosylated molecule or after later injections.

For both Adcitmer (curve slope: 0.13±0.22) and aglycosylated Adcitmer (curve slope: −3.98±0.62) treated mice, we observed a reduced tumor growth when compared with the control group (curve slope: 56.35±4.25; p=0.0002 and p<0.0001, respectively, figure 4A) which was more pronounced for aglycosylated Adcitmer (Adcitmer vs aglycosylated Adcitmer, p=0.03) resulting even in shrinkage of the tumors.

In accordance with the size measurements, autopsy revealed a significantly reduced tumor weight in animals treated with aglycosylated Adcitmer and Adcitmer (median: 0.03 g, range: 0–0.06 and 0.15 g, range: 0.1–0.41; respectively; p=0.0096) compared with the control group (median: 3.31 g, range: 2.27–3.65, p=0.0020 and p=0.0034, respectively; figure 4B). With these data, we also confirmed a significant increase in antitumor effect for the aglycosylated Adcitmer compared with WT (wild-type) Adcitmer (p=0.0096; figure 4B). No metastasis was detected, and no weight loss was observed during the experiment (Data not shown).

In summary, our results suggest that aglycosylation of Adcitmer, by preventing binding through Fc-receptor, leads to greater selectivity of the antibody for the MCC cells allowing a reduction of unspecific toxicities on immune cells, in particular on NK cells. This greater selectivity may result in a theoretically higher biodisponibility of the molecules in vivo since an improved antitumor effect could be observed in mice.

DISCUSSION

CD56 is an attractive cell surface target expressed by various hematologic malignancies[30] and solid tumors including myeloma,[31 32] SCLCs,[33 34] sarcomas and pediatric neoplasms.[35–37] As such, the CD56 targeting ADC LM has been evaluated in clinical trials but presented severe toxicities, notably neutropenia when used in combination with etoposide/cisplatin, leading to the discontinuation of this product. In this context, the aim of the present study was to optimize Adcitmer,

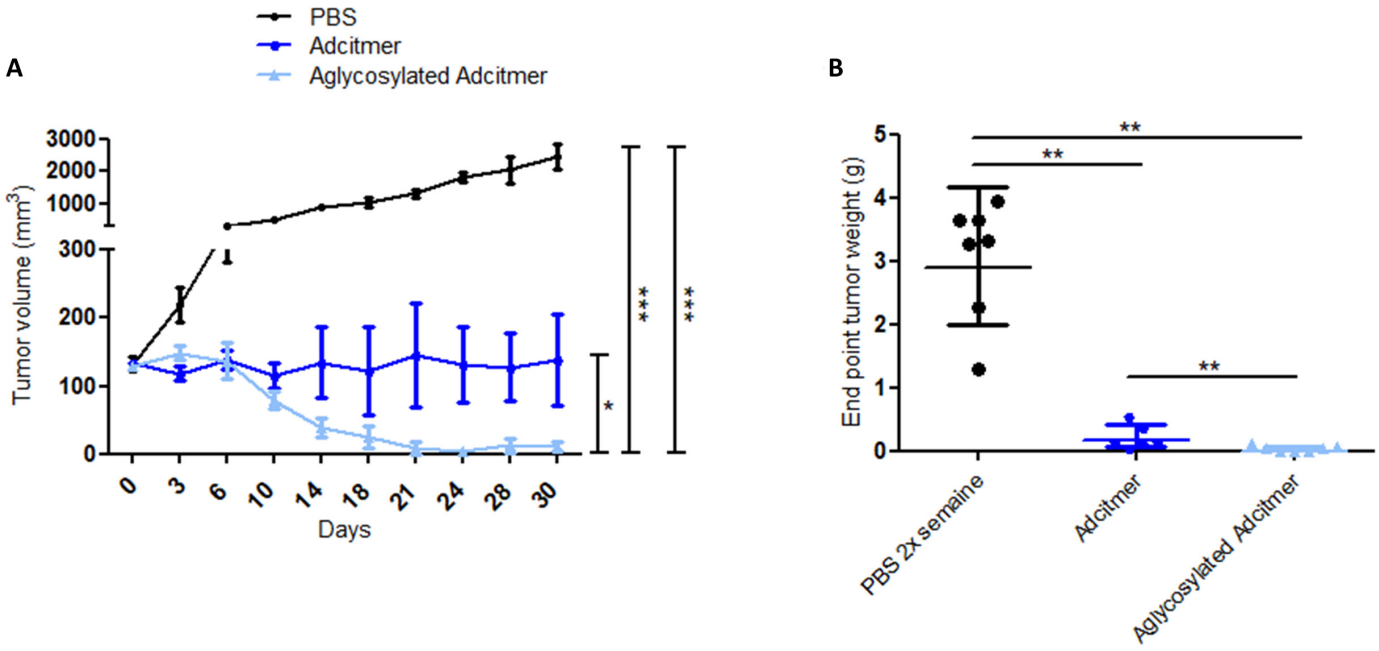

Figure 4 Both Adcitmer and aglycosylated Adcitmer efficiently suppress tumor growth in a Merkel cell carcinoma xenograft mice model. (A) Mean tumor volume curves±SEM during the experiment. Each point represents mean tumor volume in PBS, aglycosylated Adcitmer and Adcitmer. Intravenous injections were done twice a week at 5 mg/kg (each mouse received nine injections in 1 month). (B) Endpoint tumor weights in the experimental and control groups. Horizontal lines are means±SEM. PBS, phosphate buffered saline.

a CD56-targeting immunoconjugate with improved safety profile in comparison to LM to increase the therapeutic window and improve the therapeutic performance.

We demonstrated non-specific binding of the unconjugated antibody lorvotuzumab to CD56 KO cells while no such interaction was detected for m906, the antibody used to generate Adcitmer. These findings might partially explain off-target effects observed in patients treated with LM. More importantly, we confirmed that lorvotuzumab and, to a lesser extent, m906 were both toxic on neutrophils, an immune population without CD56 expression. As expected, aglycosylation avoids FcγR recognition and, interestingly, reduces toxicity on immune cells. Accordingly, while a cytotoxic effect on CD56-expressing NK cells was evidenced for lorvotuzumab and m906, aglycosylation of the m906 limited this effect without impairing specific cytotoxicity on MCC tumor cells, which is in line with a report that these cells are resistant to ADCC.[12]

Interestingly, evaluation of aglycosylated Adcitmer in MCC xenograft immunocompromised mice model in comparison to the glycosylated molecule demonstrated that aglycosylated Adcitmer reduced tumor growth more efficiently with even complete regressions in three of seven mice. To note, although mice currently represent the only animal model available for MCC with some limitations. Notably, in the present study, Adcitmer was investigated in an immunocompromised mice model, with defective NK cells, that does not allow evaluating the impact of such a product on this class of immune cells. Most importantly, in contrast to humans, a carboxylesterase 1c responsible for ValCitPAB linker-MMAE instability is present in mice[38 39] with the consequence of poor pharmacokinetics and unspecific toxicities of the free payload and may affect the specific antitumor growth effect. Indeed, when we assessed the stability of aglycosylated Adcitmer in plasma of mice, rats, non-human primates and humans after 24 hours, a significant MMAE release was only detectable in the mouse plasma (Data not shown). As carboxylesterase 1c is not present in humans, improved plasmatic stability of Adcitmer will be expected.

Another determinant of the therapeutic performance of ADCs is a potential bystander effect.[40] For example, in HER2-positive breast cancer, trastuzumab emtansine does not provide bystander killing while trastuzumab deruxtecan does. Hence, the latter ADC is effective in patients with metastatic breast cancer with low HER2 expression.[41] Similarly, in CD30-expressing Hodgkin's lymphoma or anaplastic large-cell lymphoma, the benefit of the bystander effect induced by brentuximab vedotin has been demonstrated in the case of heterogeneous CD30 expression.[42] In the present study, we could show that aglycosylated Adcitmer can induce a bystander effect while LM cannot (online supplemental figure 2). Thus, an Adcitmer bystander effect could also enhance its therapeutic efficacy in case of heterogeneous expression of the target antigen.

Interestingly, aglycosylated therapeutic antibodies have already been approved for clinical use.[43] The major goal of developing this antibody type is the reduction

of adverse clinical reactions caused by cytokine release through impaired binding of antibody to complement or Fc receptors.[44] In contrast to pure antibody-based therapy, until now, no approved ADC is manufactured with an aglycosylated antibody, but some are currently under preclinical investigation.[45]

To conclude, aglycosylated Adcitmer appears as a promising therapeutic option in preclinical models of MCC with reduced toxicity against immune cells. Therefore, aglycosylated Adcitmer might be evaluated in humans as an alternative treatment or in combination with immunotherapy for patients with advanced MCC and other CD56-positive malignancies.

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Contributors ADr designed and performed the experiments/calculation, analysed the data and wrote the original manuscript in consultation with TK and AT. LD and CE assisted in the design and helped to perform in vivo experiment. PG and VL contributed to the design of XTT and in vivo experiment. SK, MF, CD and VG-G designed experiment on the immune cells part and contributed to the interpretation of the results. CBB performed the bioconjugation process of the antibodies to produce ADCs. FB and NA performed the production of the antibodies in ExpiCHO. RH, DS, SG, VG-G, ADe and MCV-M critically reviewed the study proposal and served as scientific advisors. TK and AT supervised the project. All authors discussed the results and contributed to the final manuscript. The guarantor of the study is AT.

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