Chemically defined antibody- and small molecule-drug conjugates for in vivo tumor targeting applications: A comparative analysis

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Chemically-defined antibody- and small molecule-drug conjugates for *in vivo* tumor targeting applications: a comparative analysis

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Supporting Information Placeholder

ABSTRACT: We present the first direct comparative evaluation of an antibody-drug conjugate and of a small molecule-drug conjugate for cancer therapy, using chemically-defined products which bind with high-affinity to carbonic anhydrase IX, a marker of tumor hypoxia and of renal cell carcinoma.

Conventional cancer chemotherapeutic agents do not preferentially localize at the tumor site.[1-4] This pharmacokinetic limitation often contributes to the onset of toxicity and prevents dose escalation to therapeutically-active regimens.[5] In an attempt to improve the therapeutic index of cancer chemotherapy, monoclonal antibodies and small organic ligands have been proposed as delivery vehicles of cytotoxic compounds, allowing the construction of antibody-drug conjugates (ADCs)[6-8] and small molecule-drug conjugates (SMDCs).[9] Four ADC products have gained marketing authorization for cancer therapy[10-14], while SMDCs are still under investigation in clinical trials.[15]

Monoclonal antibodies can recognize their molecular target with exquisite specificity, but their penetration into solid tumor masses can be suboptimal.[16] ADCs have high cost-of-goods and their long circulatory half-life may cause premature drug release.[17,18] While antibodies can be routinely generated against virtually any protein antigen[19], the isolation of small organic ligands is more difficult and SMDC applications have so far been limited to a small number of targets (e.g. Folate Receptor, Prostate-Specific Membrane Antigen, Somatostatin Receptors and Carbonic Anhydrase IX).[20-23] In principle, peptides could also be considered as ligands for pharmacodelivery applications, provided that they display acceptably low kidney uptake values.[22,24]

Here we report the first comparative analysis of two chemically-defined ADC and SMDC products, directed against the same molecular target. Carbonic anhydrase IX (CAIX) is a cell membrane-protein overexpressed in tumor hypoxia and in certain malignancies, including renal cell carcinoma, colorectal, urothelial, lung, stomach, pancreas, breast, head and neck, ovaries, brain and cervix cancer.[25,26]

CAIX has been targeted in clinical trials with radionuclide conjugates[27,28] and with an ADC product.[29] A growing body of evidence indicates that ADCs and SMDCs can be efficacious even in the absence of ligand internalization, if drugs are efficiently released within the tumor mass by a number of different extracellular proteases or by the reduction of disulfide linkers.[30-37] Indeed, experimental evidence from various laboratories indicates that CAIX does not efficiently internalize upon ligand binding.[23,34,38]

For the development of an ADC product against CAIX, we used a high-affinity monoclonal antibody, isolated from a phage display library [**Supporting Information**]. The SMDC product was based on an acetazolamide derivative with sub-nanomolar dissociation constant to CAIX, recently isolated from a DNA-encoded chemical library.[39] For both agents, we used the linker-payload of Adcetris™, an approved ADC product[40,41], featuring a cleavable Val-Cit dipeptide, a self-immolating spacer and monomethyl auristatin E (MMAE) as cytotoxic drug [**Figure 1**]. In order to generate chemically defined products with drug-antibody ratio (DAR) of 2, antibodies were used in human IgG1 format, in which three cysteine residues in the hinge region had been mutated to serines, thus permitting a site-specific coupling with maleimido derivatives[42,43] [**Figure 1**]. The SMDCs featured a drug-ligand ratio (DLR) of 1.

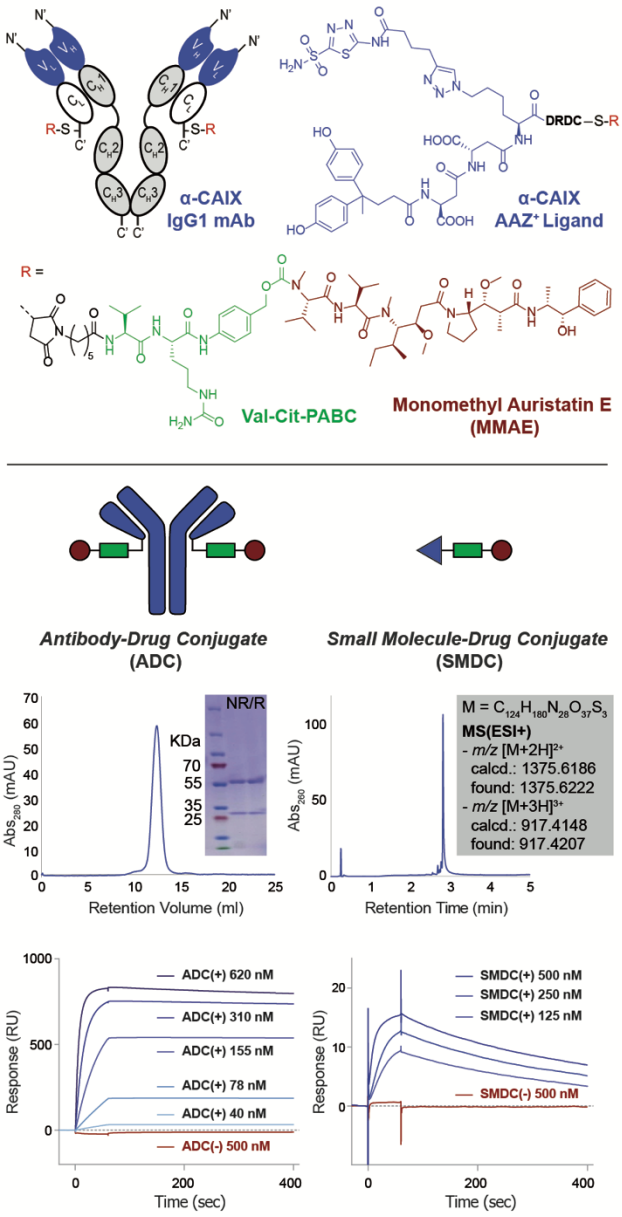

Figure 1. Chemical structures and biochemical characterization of anti-CAIX ADC and SMDC products. Ligand-and-linker-payload structures, as well as the site of conjugation are indicated. Size exclusion chromatography profile and SDS-PAGE relative to the CAIX-specific ADC(+) product. Lanes NR and R represent the final ADC in non-reducing and reducing conditions, respectively. SPR analysis of ADC(+) and the negative control ADC(-) for their binding to recombinant human CAIX. Sensograms are referred to different concentration of the conjugates. Liquid chromatography and mass spectrometry analysis of SMDC(+). SPR sensorgrams of a serial dilution of SMDC(+) and SMDC(-) against recombinant human CAIX. Fitting of sensorgrams related to ADC(+) and SMDC(+) allowed calculation of the corresponding apparent binding constants: $k_{on,SMDC(+)}$ = 3.4 10^5 $M^{-1}s^{-1}$, $k_{off,SMDC(+)}$ = 3.4 10^{-3} s^{-1}, $K_{D,SMDC(+)}$ = 10 nM; $k_{on,ADC(+)}$ = 1.7 10^5 $M^{-1}s^{-1}$, $k_{off,ADC(+)}$ = 2.2 10^{-5} s^{-1}, $K_{D,ADC(+)}$ = 0.13 nM. The BIAcore methodology may under-estimate K_D values for antibodies in homobivalent IgG format against bivalent antigens (due to a chelate binding mode) and to over-estimate K_D values for small organic ligands with very high k_{on}, due to limitation of diffusion speed within the microsensor chip hydrogel.

Methodologies to assess product purity and identity included SDS-PAGE analysis, gel-filtration and mass spectrometry for the ADCs, while the SMDCs were characterized by UPLC and mass spectrometry. Binding to the cognate CAIX antigen was studied by BIAcore analysis [**Figure 1** + **Supporting Information**]. Two structurally-related compounds were used as negative controls, as they featured ligands of irrelevant specificity in the mouse. The KSF antibody, specific to hen egg lysozyme, was used to generate an ADC product [ADC(-)], using an identical immunoglobulin format as for the anti-CAIX agent. Omission of the acetazolamide moiety in the small ligand structure led to a drug conjugate [SMDC(-)], devoid of any detectable CAIX binding [**Figure 1** + **Supporting Information**].

In order to assess the tumor-homing properties of the anti-CAIX antibody and small organic ligands, two experimental methodologies were used. Radiolabeled preparations were administered intravenously to mice bearing subcutaneous SKRC-52 tumors and the percent of injected dose per gram (%ID/g) was assessed by organ counting at relevant time points. The anti-CAIX antibody exhibited an unfavorable tumor/blood distribution ratio 48 hours after the injection, while the small organic ligand showed a substantially higher tumor uptake (~40% ID/g) and a tumor/blood distribution ratio of ~100:1 [**Figure 2A** + **Supporting Information**]. The results obtained with AAZ^+ were clearly superior to the ones obtained with monovalent acetazolamide or with homobivalent acetazolamide derivatives.[33,44] In a second experiment, the two anti-CAIX agents were administered to tumor-bearing mice and the relative uptake in relevant organs was assessed by fluorescence microscopy procedures [**Figure 2B**]. Twenty-four hours after administration, the antibody exhibited a patchy perivascular uptake in tumor cells, similar to what had previously been reported for trastuzumab in breast cancer models[16], while the small organic ligand exhibited a homogenous uptake in the neoplastic mass already after 1 hour (targeting results are shown at different time points, since the antibody clears much more slowly from circulation). The anti-CAIX antibody exhibited an undesired targeting of heart tissue, while the small ligand had a residual accumulation in kidney and lung. The results obtained with radioactive and

fluorescence detection were in good agreement [**Figure 2B** + **Supporting Information**].

Therapy experiments were performed at equimolar doses of cytotoxic agent in nude mice bearing human SKRC-52 tumors, a subcutaneous xenograft model of kidney cancer that does not efficiently metastasize *in vivo* after implantation. The SMDC products were administered at 250 nmol/Kg, while ADCs were injected at 125 nmol/Kg, as they featured a DAR of 2 [**Figure 3**]. Tumors grew rapidly in mice treated with saline. The CAIX-targeted SMDC product [SMDC(+)] exhibited a potent tumor-growth retardation, while the negative control counterpart did not slow down tumor growth. ADC(+) was potently active in this cancer model, but (like the SMDC product) did not result in complete tumor eradication.

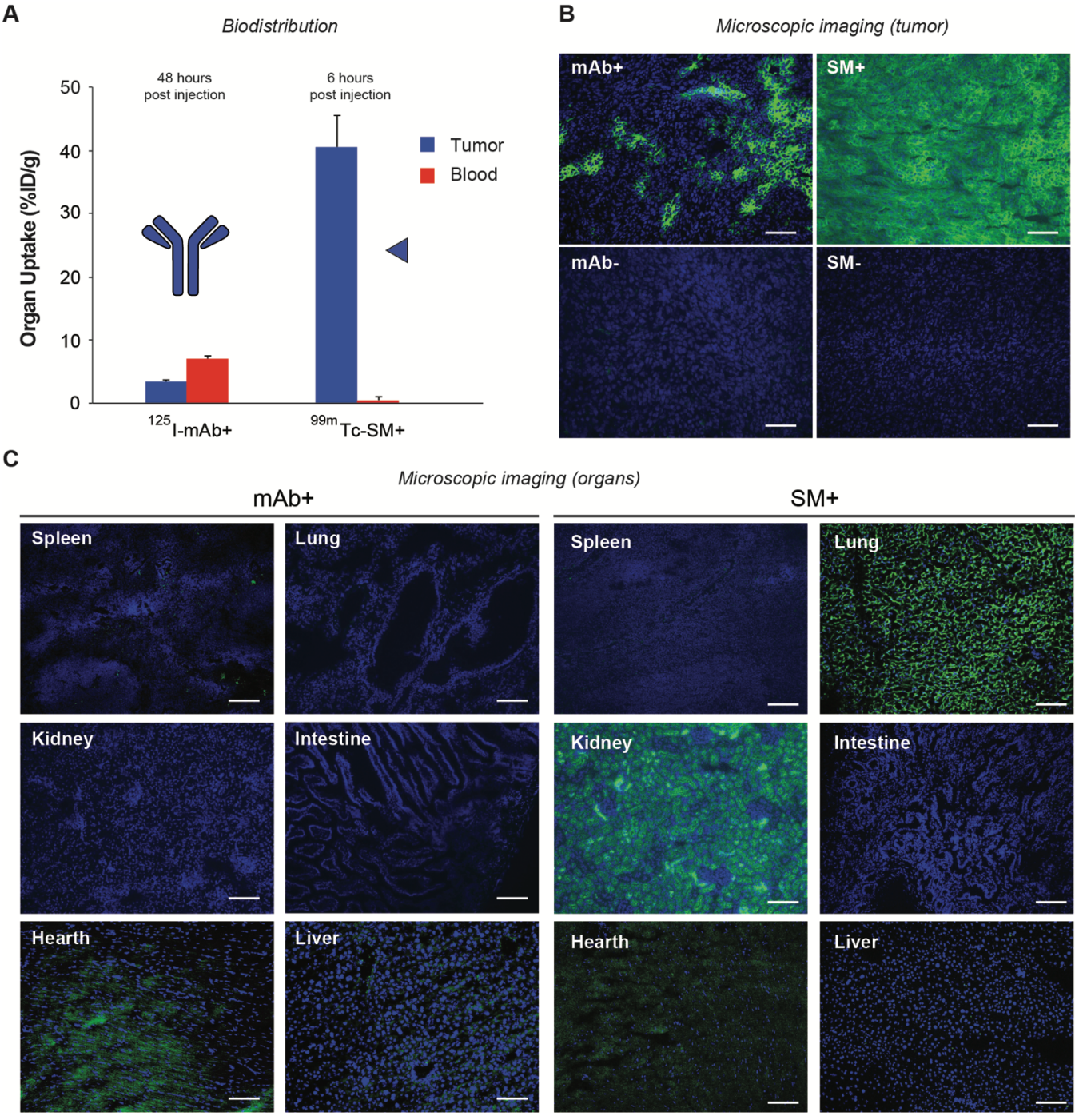

Figure 2. Evaluation of the tumor-targeting performance of the anti-CAIX XE114 antibody (mAb+) and the small ligand AAZ⁺ (SM+) against human renal cell carcinoma cells SKRC-52 xenografted in mice. (A) Quantification of ligand uptake in tumor and blood after administration of radiolabeled preparations of IgG(XE114) (mAb+) and of a radiolabeled derivative of AAZ⁺ (SM+). Microscopic distribution of IgG(XE114) (mAb+) and of a fluorescently labeled derivative of AAZ⁺

(SM+) in SKRC-52 tumors (B) and in healthy organs (C) after IV administration. Images related to mAb+ and SM+ products were taken 24 hours and 1 hour post injection, respectively. mAb- and SM- relate to the corresponding negative controls. Green = Ligand (mAb+ or SM+); Blue = DAPI staining. Scale bar = 100 μm.

Moreover, the difference in activity between positive- and negative-control ADCs was minimal. All treatments were well tolerated, even though ADC(+) led to a transient loss of 5% body weight.

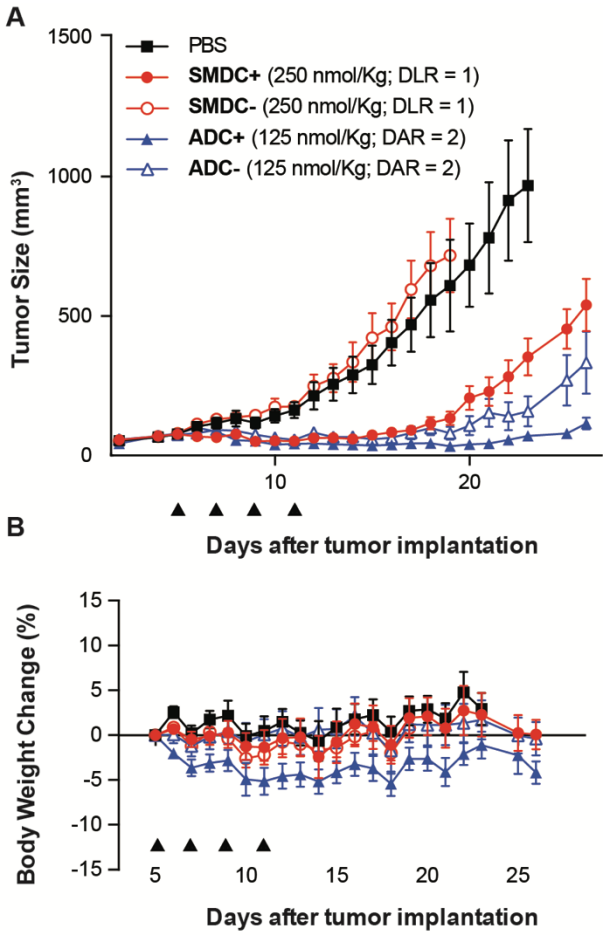

Figure 3. Comparative therapeutic analysis of anti-CAIX antibody-drug conjugate ADC(+) and small molecule-drug conjugate SMDC(+) in BALB/c nu/nu mice bearing SKRC-52 xenografts. In the experiment, ADC(-) and SMDC(-) derivatives devoid of the targeting moieties were used as negative controls. (A) Changes in tumor volume for different treatment groups. (B) Body weight changes experienced by the animals during the therapy experiment. The arrows indicate intravenous (i.v.) administration of the corresponding agent. DLR = drug-ligand ratio; DAR = drug-antibody ratio.

The experiments indicate that both ADC and SMDC products can mediate a potent anti-tumor effect in tumor-bearing mice, when used at the same molar dose. The main limitations for ADC technology may be associated with a sub-optimal tumor uptake, essentially limited to perivascular cancer cells.[16,45,46] By contrast, the small CAIX ligand exhibited an efficient and homogenous targeting of the neoplastic mass. However, small ligands (including the one used in this study) are often efficiently filtered via the renal route and may display an undesired uptake in the kidney interstitium. A residual uptake of the CAIX ligand in stomach and lung was observed at early time points, but its magnitude was substantially lower compared to the one in tumors [**Supporting Information**]. The SMDC product exhibited an excellent discrimination, relative to its negative control counterpart, both in biodistribution and in therapy studies. However, the therapeutic activity was slightly inferior compared to the ADC. This observation was somewhat unexpected, in light of the biodistribution results of **Figure 2**. It is likely that the anti-CAIX ADC product displays its activity mainly by a slow release of the highly-potent MMAE cytotoxic payload, as a strong anti-cancer activity was also observed for the anti-lysozyme ADC(-) negative control. Interestingly, charged analogues of MMAE do not exhibit comparable therapeutic activity *in vivo*, when coupled to non-internalizing ligands.[47]

The Val-Cit-based linker-payload combination used in this study is the same as the one used in the clinically-approved Adcetris™ ADC product. It is possible that the SMDC agent may benefit from a careful tuning of the velocity of payload release.[48] Considering that the high-affinity acetazolamide-based CAIX ligand described in this article displays a tumor/blood distribution ratio of ~100:1 six hours after i.v. injection, a more labile linker may increase the rate of tumor cell damage and may therefore be more active. An interplay between dose rate and therapeutic activity has previously been reported for radionuclide-based therapeutics.[49,50] It also remains to be seen whether ADCs and SMDCs still exhibit a comparable therapeutic activity, when those products are directed against a target which internalizes well.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website.

Detailed experimental procedures and Supplementary Figures (PDF)

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Notes

D.N. is a co-founder and shareholder of Philogen (www.philogen.com), a Swiss-Italian Biotech company that operates in the field of ligand-based pharmacodelivery.

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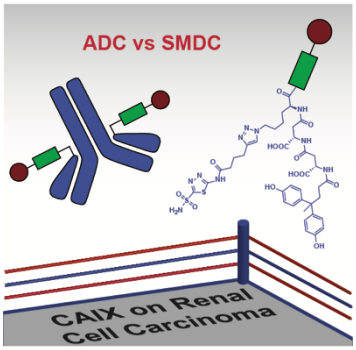

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