Cancer Therapy

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A Small-Molecule Drug Conjugate for the Treatment of Carbonic Anhydrase IX Expressing Tumors**

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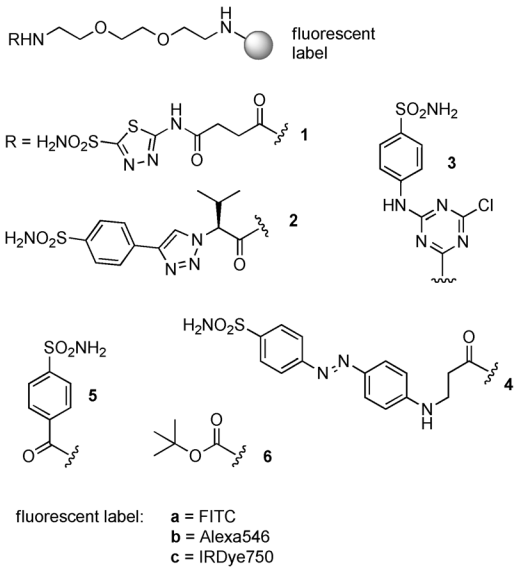
Abstract: *Antibody–drug conjugates are a very promising class of new anticancer agents, but the use of small-molecule ligands for the targeted delivery of cytotoxic drugs into solid tumors is less well established. Here, we describe the first small-molecule drug conjugates for the treatment of carbonic anhydrase IX expressing solid tumors. Using ligand–dye conjugates we demonstrate that such molecules can preferentially accumulate inside antigen-positive lesions, have fast targeting kinetics and good tumor-penetrating properties, and are easily accessible by total synthesis. A disulfide-linked drug conjugate with the maytansinoid DM1 as the cytotoxic payload and a derivative of acetazolamide as the targeting ligand exhibited a potent antitumor effect in SKRC52 renal cell carcinoma in vivo. It was furthermore superior to sunitinib and sorafenib, both small-molecule standard-of-care drugs for the treatment of kidney cancer.*

The targeted delivery of highly potent cytotoxic agents into diseased tissues has emerged as a promising strategy for the treatment of cancer and other serious conditions. By attaching a therapeutic effector through a cleavable linker to a ligand specific to a marker of disease, the effector preferentially accumulates and acts at the intended site of action, thus increasing the effectively applied dose while reducing side effects. To date, monoclonal antibodies have been considered as the ligands of choice[1,2] and, indeed, research in the field of antibody–drug conjugates (ADCs) has led to the recent approval of two ADCs for applications in oncology: brentuximab vedotin and trastuzumab emtansine.[3]

In spite of these very encouraging developments, the limitations of using antibodies for drug-delivery applications are becoming increasingly clear.[4] Antibodies are large macromolecules and thus often have difficulties penetrating deeply into solid tumors.[5] In addition, they can be immunogenic[6] and typically long circulation times[7] can lead to premature drug release and undesired side effects. Moreover, the production of ADCs is expensive, reflecting the need for the clinical-grade manufacturing of antibodies, drugs, and the resulting conjugates.[4]

The use of smaller ligands as delivery vehicles such as peptides or small drug-like molecules could potentially overcome some of the above-mentioned problems. Their reduced size should aid tissue penetration, and they should be nonimmunogenic and amenable to classic organic synthesis thus reducing manufacturing costs.[4,8] Indeed, the favorable properties of drug conjugates that use folic acid[9] and those with ligands against prostate-specific membrane antigen (PSMA)[10] as delivery vehicles have been demonstrated and a folate conjugate has recently entered Phase III clinical studies.[11] In spite of growing interest from both academia and industry, the field is still in its infancy as few ligands have been



Scheme 1. Chemical structures of ligand–linker–dye conjugates synthesized for in vitro binding and in vivo targeting studies. FITC = fluorescein isothiocyanate.

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studied systematically and only a small number of targets have been tested successfully.

Here, we report the design and characterization of a family of novel small-molecule drug conjugates (SMDCs) targeting carbonic anhydrase IX (CAIX) expressing solid tumors. CAIX is over-expressed in many different forms of cancer such as glioblastoma,[12] colorectal,[13] and breast cancer[14] as a marker of hypoxia, and thus represents a very attractive antitumor target.[15] In renal cell carcinoma it is often constitutively expressed and is among the best-characterized cell-surface markers of this disease.[16] Other carbonic anhydrase isoforms fulfill diverse physiological roles in the body. Whilst many are intracellular (e.g., the abundant CAII) some are also extracellularly located (e.g., CAXII).[17,18]

Based on known structure–activity relationship data[19–21] a series of high-affinity CAIX ligand-linker-dye conjugates **1a**–**5c** (Scheme 1) was prepared. The dissociation constants of **1a**–**3a** and **5a** towards recombinant CAIX were determined by fluorescence polarization (FP, Figure 1a). Flow cytometry experiments indicated ligand-dependent binding of Alexa546 and IRDye750 conjugates **1b** and **1c**–**5c** to CAIX-positive cells (Figure 1b, Figure 1 in the Supporting Information (SI)), but not to control cell lines lacking CAIX (Figures 2 and 3 (SI)). In contrast to previous reports that suggested receptor-based internalization of CAIX-specific ligands,[22–25] acetazolamide-based fluorophore conjugates were found to preferentially bind to the cell membrane of kidney cancer cells without efficient internalization (Figure 1c, Figure 5 (SI)), while the same cells were not stained by fluorophores lacking the tumor-homing moiety (Figure 1d). These results collectively suggest that fluorescent probes **1a–c** derived from the approved antiglaucoma drug acetazolamide (AAZ) were high-affinity CAIX binders ($K_D = 12.6$ nM for **1a**) and, thus, potentially suitable for pharmacodelivery applications.

We thus investigated the potential of **1b** and **1c** to to reach tumors in vivo (Figure 2). An intravenous dose of 3 nmol

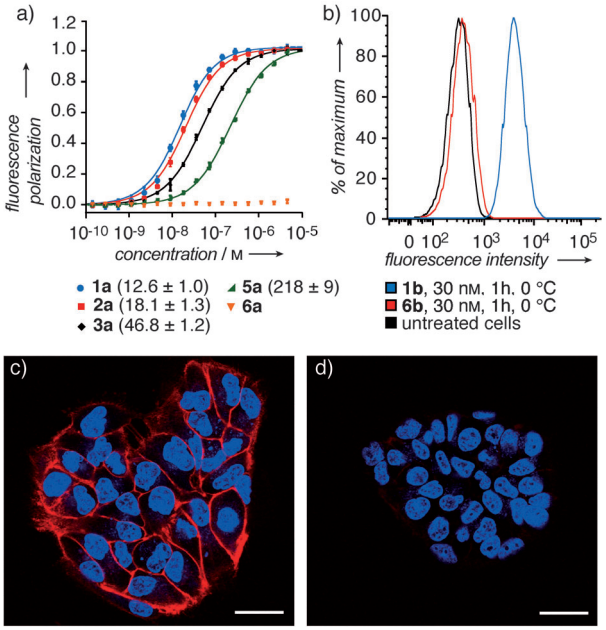


Figure 1. In vitro characterization of ligand–linker–dye conjugates a) Determination of the K_D values of **1a**–**3a**, **5a**, and **6a** towards recombinant CAIX extracellular domain in vitro by fluorescence polarization. The K_D value of the diazo ligand **4a** could not be determined due to its strong absorption at 495 nm. Data points are given as averages of three experiments. Error bars indicate standard deviations. K_D values are given in brackets in nM ± standard errors of fit. b) Flow cytometric analysis of the binding of Alexa546 conjugates **1b** and **6b** to CAIX-expressing SKRC52 cells. Only conjugate **1b** containing the CAIX ligand binds to the cells, whereas nonbinding conjugate **6b** produces a histogram superimposable to that of untreated cells. c) Confocal microscopy image of CAIX-expressing SKRC52 cells after exposure to targeted dye conjugate **1b** (30 nM) for 1 h. The conjugate is mainly bound to the cell surface. d) Confocal microscopy image of CAIX-expressing SKRC52 cells exposed to untargeted dye conjugate **6b** (30 nM) for 1 h. No cell surface binding can be detected. Scale bar: 35 μm.

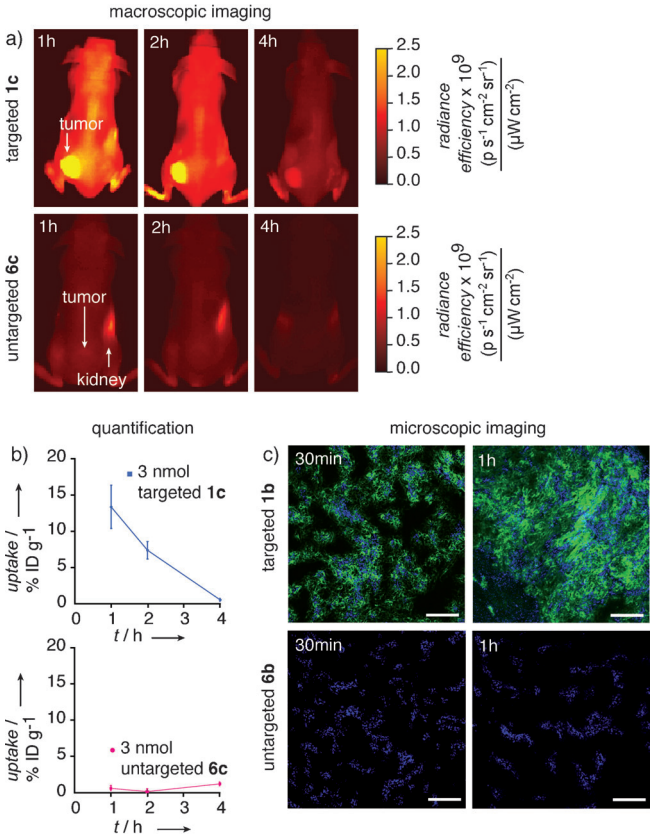


Figure 2. Evaluation of tumor-homing performance of targeted conjugates **1b** and **1c** compared to that of untargeted controls **6b** and **6c**. a) Whole-animal near-infrared fluorescence images of a representative mouse 1, 2, and 4 h after intravenous administration of 3 nmol targeted ligand–IRDye750 conjugate **1c** or **6c**. A preferential accumulation of construct **1c** in the tumor can be observed. Without the targeting ligand the dye conjugate **6c** can only be seen in the kidneys as a major route of excretion. b) Quantification of targeted conjugate **1c** or **6c** in homogenized samples of tumors extracted 1, 2, and 4 h after intravenous administration of 3 nmol dye conjugate. Error bars indicate standard deviations. Averages of three experiments are shown. c) 10 μm section of tumor 30 min and 1 h after intravenous administration of 50 nmol targeted ligand–Alexa546 conjugate **1b** or untargeted **6b**. Penetration of the fluorescent dye conjugate (green) into the tumor increases over time. Areas in the immediate surrounding of blood vessels were stained by a 5 min perfusion of the animal with Hoechst 33342 (blue) prior to organ extraction. Scale bar: 200 μm. The untargeted conjugate does not reach the tumor in detectable levels.

AAZ-based conjugate **1c** preferentially accumulated in subcutaneous CAIX-expressing SKRC52 tumors[26] in nude mice (Figure 2a, Figures 7 and 8 (SI)). Intravenous administration of 3 nmol of **2c** resulted in moderate visible tumor accumulation after 1 h (Figure 8 (SI)), but the tumor-to-background ratio was only poor. By contrast, conjugates **3c**–**5c** and untargeted dye conjugate **6c** did not exhibit a preferential tumor homing (Figure 2a, Figure 8 (SI)). Since **1a** has the lowest K_D value followed by **2a**, these results suggest that binding affinity contributes to efficient tumor targeting. As the best cancer localization results were obtained with **1c**, we decided to quantitatively evaluate the biodistribution of this compound, comparing it to the tissue distribution properties of the untargeted dye **6c**.

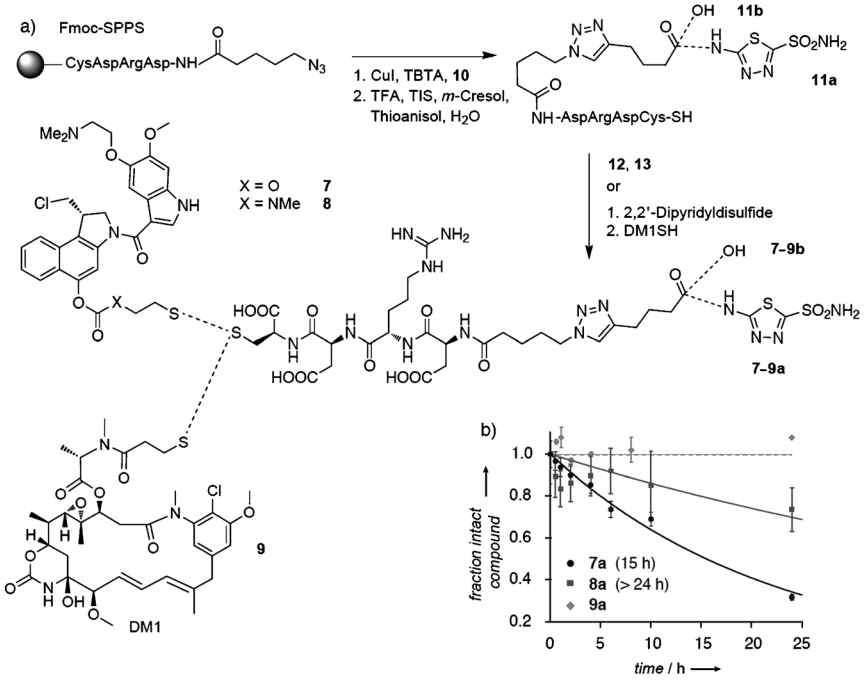
Accumulation of **1c** in the tumor was rapid and efficient with $(13.4 \pm 3.0)\%$ of injected dose per gram of tissue (% ID g^{-1}) after only 1 h (Figure 2b, Figures 10 and 11 (SI)). This result compares favorably with previous work on the antibody-based targeting of CAIX-expressing tumors, where only markedly lower tumor uptake values (a maximum of $(2.4 \pm 0.2)\%$ ID g^{-1}) could be detected.[27] In our case, the dye conjugate **1c**, however, progressively dissociated from the tumor (residence half-life $t_{1/2} \approx 1$ h), suggesting that an improvement of CAIX binding affinity may further contribute to efficient tumor targeting performance.

A tumor-to-blood ratio of 13.8:1 was observed 1 h after intravenous injection of **1c** (Table 2 (SI)) and further improved to 79.2:1 after 4 h. Tumor-to-organ ratios for excretory organs ranged between 0.2:1 for liver and 1.4:1 for kidneys after 1 h (Tables 1 and 2 (SI)) but a high level of selectivity was observed for other organs (e.g., 27.6:1 for tumor to heart after 1 h, Table 2 (SI)). AAZ is a CA ligand with broad isoform selectivity,[19] but the generation of derivatives containing multiple charges may limit their tissue distribution to the extracellular space and thus restrict binding to extracellular membrane-associated CAs. Importantly, tumor targeting was clearly dependent on the CAIX-binding moiety, as revealed by strikingly different areas under the curve of tumor uptake over time (Figure 2b) and by the fact that the AAZ-based targeted dye conjugate **1c** had a 22-fold higher tumor accumulation at 1 h than the nontargeted dye **6c** (Table 1 (SI)). Assuming that **6c** is a good model for the tissue distribution of "naked" (i.e., untargeted) anticancer agents, this comparison highlights the potential impact of the ligand-based drug delivery of therapeutically relevant doses of drugs into neoplastic masses.

To gain an understanding of the microscopic distribution of small ligand-based targeted dye conjugates inside tumors, **1b** and **6b** were intravenously injected into SKRC52 tumor-bearing mice. Already after 30 min, **1b** (Figure 2c) had diffused outside the immediate surrounding of the blood vessels stained by perfusion of the animal with Hoechst 33342 shortly before tumor extraction. Nontargeted dye conjugate **6b** did not reach the tumor in detectable levels (Figure 2c). As a further indication of targeting selectivity, fluorescence was restricted to CAIX-positive tissues and excretory organs (Figure 13 (SI)).

The promising tumor-targeting performance of AAZ derivatives motivated us to investigate the therapeutic activity of conjugates comprising a linker cleavable in the extracellular space and a potent cytotoxic payload. We focused on conjugates **7a**,**b** and **8a**,**b** with a duocarmycin derivative as the payload and on the DM1 conjugates **9a**,**b**, since these cytotoxic drugs are actively being used for the development of anticancer antibody–drug conjugates.[28,29] Since CAIX did not internalize efficiently in our hands (Figure 1c), we hypothesized that the disulfide bond would be cleaved by reducing agents in the tumor extracellular space (e.g., glutathione liberated by dying tumor cells). Subsequently, the drug would diffuse into the neighboring neoplastic cells. A similar process has been proposed for non-internalizing antibody–drug conjugates.[30]

The syntheses of the drug conjugates **7a**–**9a** are outlined in Scheme 2a. For all conjugates, a charged peptide spacer related to a previously described linker was used in order to improve water solubility.[11] The targeting ligand was attached



Scheme 2. Structure, synthesis, and stability of drug conjugates. a) A Cu^{I}-catalyzed azide–alkyne cycloaddition was used to couple the targeting ligand **10** to the charged linker, which was prepared by fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS). Therapeutic payloads were installed by disulfide exchange. b) Hydrolytic stability of drug conjugates in PBS pH 7.4 at 37°C as determined by mass spectrometry (**7a** and **8a**) and high-performance liquid chromatography (**9a**). The carbamate derivative of duocarmycin conjugate **8a** ($t_{1/2} > 24$ h) was found to be more stable than the carbonate ($t_{1/2} = 15$ h). DM1 conjugate **9a** was found to be hydrolytically stable. TBTA = tris(benzyltriazolylmethyl)amine, TFA = trifluoroacetic acid, TIS = triisopropylsilane.

using an Cu^{I}-catalyzed alkyne–azide cycloaddition since direct peptide coupling of the AAZ succinic acid derivative as in **1a** consistently failed. For the duocarmycin conjugates **7a**,**b** and **8a**,**b**, the residual carbonate or carbamate linker was designed to self-immolate upon disulfide cleavage, yielding the active drug.[11,31,32] Conjugates **7b**, **8b**, and **9b** lacked AAZ as the targeting ligand and were used as negative controls.

Drug conjugates bound CAIX in vitro with low nanomolar affinities whilst control compounds only interacted weakly ($K_D > 1$ μM), as tested by competitive fluorescence polarization against **1a** (Figure 14 (SI)).[33] As expected, the carbonate **7a** ($t_{1/2} = 15$ h) was less stable in PBS at 37 °C than the carbamate **8a** ($t_{1/2} > 24$ h). No decomposition was observed for the DM1 conjugate **9a** under the same conditions (Scheme 2b). The stability of **7a** and **8a** was reduced in mouse serum in vitro[34] ($t_{1/2} = 43$ and 61 min respectively, Figure 15a (SI)), but occurred in a time range compatible with the preferential accumulation of the AAZ conjugates at the tumor site (Figure 2b). The DM1 conjugate **9a** was significantly more stable ($t_{1/2} = 20$ h, Figure 15b (SI)). The conjugates were cytotoxic when tested in an in vitro assay (Figures 16 and 17 (SI)).

The therapeutic activity of the conjugates **7**–**9a**,**b** was tested in mice bearing subcutaneous SKRC52 tumors. Drugs were administered at a recommended dose, which was experimentally determined by dose escalation studies (Figures 18 and 19 (SI)). Equimolar amounts of untargeted conjugates **7b**, **8b**, and **9b** together with AAZ were used as negative controls.

The therapeutic results obtained with the duocarmycin–AAZ conjugates only indicated a modest tumor growth inhibition effect (Figure 3a). Nevertheless, targeted carbonate **7a** gave rise to statistically significant tumor growth retardation compared with that observed in mice that only received vehicle ($p < 0.0001$) and mice receiving untargeted conjugate **7b** plus equimolar amounts of AAZ ($p < 0.05$). The carbamate-based constructs **8a** and **8b** did not lead to any retardation in tumor growth. It seems reasonable that the low affinity of **8a** towards the antigen ($K_D = (40.3 \pm 2.6)$ nM versus $K_D = (7.3 \pm 0.5)$ nM for **7a**) and inefficient extracellular activation may have been partly responsible for this effect. The treatment could be performed with a weight loss lower than 15% of body weight (Figure 3b).

For the DM1 conjugate **9a**, a potent antitumor effect was observed at doses that gave only minimal toxicity (i.e., no detectable body weight loss when 7×70 nmol of DM1 conjugate **9a** was given on 7 consecutive days). During the treatment period tumors shrank and continued to reduce in volume for 7 additional days. Only 20 days after the start of treatment did tumors start to grow again, as a consequence of the mice not receiving additional drug treatment. Importantly, neither sorafenib nor sunitinib, which represent the most commonly used chemotherapeutic agents for the treatment of kidney cancer,[35] exhibited any detectable antitumor effect, in line with previous reports in different models of kidney cancer.[36] These findings suggest that the targeted delivery of potent cytotoxic agents may provide a therapeutic advantage over the current standard of care. DM1 may be a particularly suitable payload for the development of

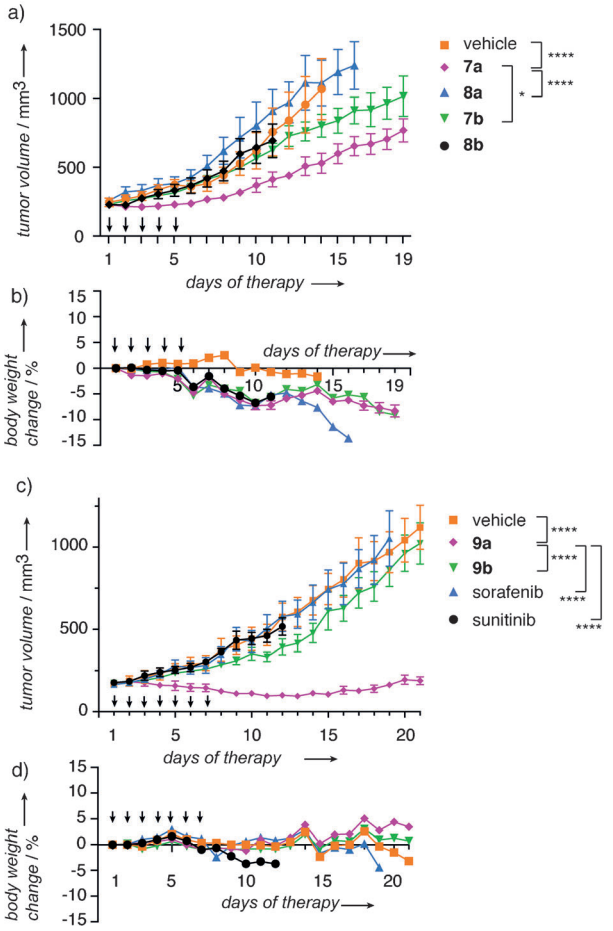


Figure 3. Therapy experiments a) Growth of SKRC52 xenografts in balb/c nu/nu mice treated 5 times on 5 consecutive days (arrows) with 4 nmol of duocarmycin derivative-based drug conjugates **7**–**8a**,**b**, or vehicle (5% DMSO in PBS pH 7.4). Untargeted conjugates **7b** and **8b** were administered with equimolar amounts of AAZ. Error bars give standard errors. b) Change of average weight of animals treated with compounds from (a); error bars give standard deviations. c) Growth of SKRC52 xenografts in balb/c nu/nu mice treated 7 times on 7 consecutive days (arrows) with 70 nmol of DM1-based drug conjugates **9a** and **9b**, or vehicle (5% DMSO in PBS pH 7.4). Untargeted conjugate **9b** was administered with equimolar amounts of AAZ. The targeted conjugate **9a** has a significantly stronger antitumor effect than the untargeted conjugate **9b** and leads to prolonged suppression of tumor growth. After 20 days, tumors started regrowing because the mice had not received any additional drug treatment. Clinically used kinase inhibitors sorafenib and sunitinib were given as further controls at a standard dose of 30 mg kg^{-1} but did not exhibit any activity; error bars give standard errors. d) Change of average weight of animals treated with compounds from (c); error bars give standard deviations. * indicates $p < 0.05$; **** indicates $p < 0.0001$. Groups were stopped when the tumor of one animal in the group reached > 2000 mm^3 in volume or weight dropped by > 15% with the exception of **9a** where all animals were still alive after 40 days. Each group consisted of five or six animals.

targeted cytotoxics, since the presence of, for example, an ester moiety in its structure may facilitate its detoxification by esterases in clearance-related organs, thus sparing healthy tissues.[37,38]

To the best of our knowledge, this is the first report of a therapeutic effect of small-molecule drug conjugates

directed against CAIX, a validated and accessible marker of renal cell carcinoma.[16] Our work demonstrates that targeting noninternalizing antigens with small-molecule conjugates can lead to a preferential product uptake at the tumor site and release of cytotoxic payloads, with a potent antitumor activity. A judicious choice of linker–payload combinations contributes to therapeutic performance, as evidenced by the different results obtained with conjugates of DM1 and duocarmycin derivatives, two widely used cytotoxic drugs for ADC development.[28,29]

Our quantitative biodistribution studies revealed the contribution of the CAIX-binding moiety to the preferential accumulation of payloads at the tumor site. By improving the affinity and isoform selectivity of CAIX-targeting ligands we expect that therapeutic performance may further be improved in the future. Importantly, similar studies could be performed using nuclear medicine techniques in cancer patients, thus bridging the translational gap between rodent models of cancer and man. We believe that our findings may facilitate the development of targeted drugs for the treatment of CAIX-expressing tumors. Some of the findings presented here may have a broader applicability for the developments of targeted cytotoxics directed against other tumor-associated antigens.

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