DRUG CONJUGATES SPECIFIC TO CARBONIC ANHYDRASE IX FOR CANCER THERAPY

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Presented by

Samuele Cazzamalli
MSc., Università degli Studi di Milano

Born on December, 16th 1990

Citizen of Italy

Accepted on the recommendation of
Prof. Dr. Dario Neri, examiner
Prof. Dr. Karl-Heinz Altmann, co-examiner

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To my Family
TABLE OF CONTENTS

I. SUMMARY ..........................................................................................................................9
   1. ENGLISH .........................................................................................................................9
   2. ITALIANO .........................................................................................................................12

II. INTRODUCTION .............................................................................................................15
   1. CONVENTIONAL CANCER CHEMOTHERAPY ............................................................16
   2. TARGETED DELIVERY OF CYTOTOXIC DRUGS .........................................................18
      2.1 Antibody-Drug Conjugates .....................................................................................18
      2.2 Small Molecule-Drug Conjugates ...........................................................................23
   3. CARBONIC ANHYDRASE IX AS TARGET FOR CANCER THERAPY .........................29
      3.1 Carbonic Anhydrase IX Expression in Solid Tumors ..............................................29
      3.2 CAIX Inhibition as a Therapeutic Anti-Tumor Strategy ...........................................31
      3.3 Carbonic Anhydrase IX as a Target for Drug Delivery ...........................................32
   4. INTERNALIZING VERSUS NON-INTERNALIZING TARGETED CYTOTOXICS ............34
   5. CYTOTOXIC PAYLOADS AND LINKER TECHNOLOGIES ........................................37
      5.1 Linkers .......................................................................................................................37
      5.2 Payloads .....................................................................................................................40
   6. FUTURE PERSPECTIVES ..............................................................................................42
   7. AIM OF THE THESIS ......................................................................................................44

III. ACETAZOLAMIDE SERVES AS SELECTIVE DELIVERY VEHICLE FOR DIPEPTIDE-LINKED DRUGS TO RENAL CELL CARCINOMA ...........................................46
   1. INTRODUCTION .............................................................................................................46
   2. RESULTS .........................................................................................................................49
      2.1 Synthesis and tumor-targeting properties of acetazolamide derivatives .......... 49
      2.2 Therapy experiments .............................................................................................54
   3. DISCUSSION ..................................................................................................................57

IV. LINKER STABILITY INFLUENCES THE ANTI-TUMOR ACTIVITY OF ACETAZOLAMIDE-DRUG CONJUGATES FOR THE THERAPY OF RENAL CELL CARCINOMA ..............................................................................60
   1. INTRODUCTION .............................................................................................................60
   2. RESULTS .........................................................................................................................63
      2.1 Synthesis and in vitro characterization of acetazolamide-based drug conjugates 63
      2.2 Therapy experiments .............................................................................................66
   3. DISCUSSION ..................................................................................................................69

V. ENHANCED THERAPEUTIC ACTIVITY OF NON-INTERNALIZING SMDCS TARGETING CAIX IN COMBINATION WITH TARGETED IL-2 ........................................72
   1. INTRODUCTION .............................................................................................................72
   2. RESULTS .........................................................................................................................75
      2.1 Synthesis and in vitro activity of acetazolamide SMDC derivatives .... 75
      2.2 Therapy experiments in SKRC-52 renal cell carcinoma model .............................79
      2.3 Therapy experiments in immunocompetent mice, bearing syngeneic CT26.3E10 tumors ..........................................................87
   3. DISCUSSION ..................................................................................................................92

VI. CHEMICALLY-DEFINED ANTIBODY- AND SMALL MOLECULE-DRUG CONJUGATES FOR IN VIVO TARGETING APPLICATIONS: A COMPARATIVE ANALYSIS ..................................................................95
   1. INTRODUCTION .............................................................................................................95
   2. RESULTS .........................................................................................................................96
I. SUMMARY

1. English

Conventional chemotherapy is still widely applied for the treatment of cancer. However, small molecules used as chemotherapeutic agents inefficiently localize to solid tumors. This unfavorable biodistribution profile, together with a non-selective mechanism of action, determines severe side effects and prevents dose escalation to therapeutically active regimens.

The active delivery of potent cytotoxic compounds to tumors has been proposed as a strategy to overcome the limitations of chemotherapy. Some antibodies and small organic ligands, capable of binding to accessible tumor-associated antigens, can accumulate at the site of antigen-positive malignancies. Antibody-drug conjugates (ADCs) and small molecule-drug conjugates (SMDCs) represent two innovative classes of biopharmaceuticals that aim at a selective delivery of cytotoxic agents to the neoplastic mass.

Clinical success of ADC products may be hindered by their slow tumor penetration and by a long circulation time, which may favor a premature drug release and cause related systemic toxicities. SMDCs have been proposed to overcome some of the limitations associated to antibody carriers. Indeed, small organic ligands can deeply penetrate solid organs and tumors already few minutes after intravenous administration, and are generally characterized by a rapid excretion from the blood circulation.

This study is focused on the generation of novel SMDCs and ADCs against carbonic anhydrase IX (CAIX), a tumor-associated membrane antigen over-expressed in the majority of renal cell carcinomas and in hypoxic tumors.

Acetazolamide (AAZ), a small organic nanomolar binder of CAIX, was used as delivery vehicle in our SMDC products. Using radiolabeled preparations of the ligand we could observe a selective accumulation in human renal cell carcinoma xenografted in nude mice. Furthermore, the tumor and organ uptake of the drug could be saturated at the highest
dose applied. The sub-cellular localization of fluorescently labeled AAZ derivatives was described by confocal microscopy, confirming that CAIX does not efficiently internalize upon binding.

With the aim to evaluate the therapeutic potential of our non-internalizing drug delivery platform we coupled two highly potent cytotoxic compounds, monomethyl auristatin E (MMAE) and the anthracycline derivative PNU-159682, to acetazolamide via a valine-citrulline cleavable linker. The resulting SMDCs showed remarkable in vivo anti-tumor activity at the MTD with a substantial inhibition of tumor growth over a prolonged period of time, in a RCC model that typically does not respond to approved drugs like sunitinib or sorafenib.

With the aim to optimize drug release for our anti-CAIX SMDC products, we generated and compared new derivatives in which acetazolamide was conjugated to MMAE through four different dipeptide linkers. Products featuring a valine-citrulline or a valine-alanine linker exhibited high serum stability and superior in vivo therapeutic activity, compared to the counterparts based on valine-arginine or valine-lysine. However, in none of the cases complete tumor eradication was possible.

To investigate the importance of the ligand affinity against CAIX, we coupled the linker-payload Vedotin (valine-citrulline-MMAE) to an affinity matured derivative of acetazolamide, named AAZ⁺, that was previously isolated by our group using the DNA-encoded chemical library technology. The novel SMDC was well tolerated and highly potent in vivo. Complete and durable cures were obtained in tumor bearing mice, when AAZ⁺-valine-citrulline-MMAE was combined with L19-IL2, a clinical stage fusion protein that delivers interleukin-2 to the tumor extracellular matrix.

Finally, we performed the first head-to-head comparison between an SMDC and an ADC product. With this purpose, we compared AAZ⁺-valine-citrulline-MMAE with a chemically defined anti-CAIX ADC. Both the SMDC and the ADC displayed a potent anti-tumor activity when administered at equimolar doses in tumor bearing mice. Importantly, and in contrast with the SMDC, the activity of the ADC product could not be uniquely related to its selectivity for CAIX. Indeed, when the anti-CAIX antibody was replaced with an irrelevant antibody used as negative
control, we observed a substantial anti-cancer activity, suggesting that (at least in part) the therapeutic effect of antibody conjugates may be due to the slow release in circulation of potent cytotoxic payloads. Our findings may be of clinical significance, since carbonic anhydrase IX is over-expressed in the majority of clear cell renal cell carcinomas and in tumor hypoxia. AAZ and AAZ\(^+\) based drug conjugates may represent a promising class of targeted cytotoxics for the treatment of CAIX-positive malignancies. Furthermore, our results give a strong rationale for the use of immunostimulatory drugs, like targeted interleukin-2, as combination partner of SMDC products.
2. Italiano

La chemioterapia convenzionale è ancora ampiamente applicata nel trattamento del cancro. Tuttavia, i composti utilizzati in come agenti chemioterapeutici si localizzano con poca efficienza in tumori solidi. Questo sfavorevole profilo di biodistribuzione, assieme ad un meccanismo di azione non selettivo, determina gravi effetti collaterali e impedisce un incremento della dose a regimi terapeuticamente attivi.

Veicolare potenti composti citotossici al sito tumorale è stato proposto come metodo alternativo atto a superare i limiti della chemioterapia classica. Alcuni anticorpi e alcuni piccoli ligandi organici, capaci di legare tumor-associated antigens, possono accumularsi in tumori solidi.

Antibody-drug conjugates (ADCs) e small molecule-drug conjugates (SMDCs) rappresentano due classi innovative di prodotti biofarmaceutici, disegnate al fine di portare selettivamente agenti citotossici alla massa tumorale.

Il successo clinico dei prodotti ADC potrebbe essere limitato dalla loro lenta velocità di penetrazione di tumori e da un'elevata emivita ematica, che potrebbe favorire il rilascio prematuro del farmaco e relativa tossicità sistemica. Prodotti SMDC sono stati proposti per superare alcuni limiti tipici degli anticorpi. Infatti, i piccoli ligandi organici possono penetrare in profondità organi solidi e tumori nel giro di pochi minuti dalla somministrazione, e sono in genere caratterizzati da una rapida escrezione dalla circolazione.

Questo studio è focalizzato sulla generazione di nuovi SMDC e ADC contro anidrasi carbonica IX (CAIX), un antigene di membrana associato a tumori sovra-espresso nella maggior parte di carcinomi a cellule renali (RCC) e in tumori in ipossia.

Acetazolamide (AAZ), un piccolo ligando organico nanomolare di CAIX, è usato come veicolo di trasporto nei nostri prodotti SMDC. Utilizzando preparazioni radioattive del ligando abbiamo osservato un accumulo selettivo in carcinomi a cellule renali umani trapiantati in topi nudi. In aggiunta, abbiamo dimostrato che l’accumulo del farmaco nei tumori e in organi sani viene saturato alla più alta dose utilizzata. La localizzazione
sub-cellulare di derivati fluorescenti di AAZ è descritta grazie alla microscopia confocale, confermando che CAIX non internalizza in modo efficiente in seguito all’interazione con suoi ligandi. Allo scopo di valutare il potenziale terapeutico della nostra piattaforma non-internalizzante di targeting, abbiamo coniugato due potenti composti citotossici, monometil auristatina E (MMAE) e l’antraciclin A (PNU-159682), ad acetazolamide tramite il linker peptidico valina-citrullina. Questi coniugati SMDC hanno dimostrato una notevole attività anti-tumorale in vivo alla dose massima tollerata con una sostanziale inibizione della crescita del tumore per un lungo periodo di tempo, in un modello di RCC che tipicamente non risponde a farmaci approvati come sunitinib e sorafenib. Allo scopo di ottimizzare la velocità di rilascio del farmaco abbiamo generato nuovi derivati nei quali acetazolamide è coniugata ad MMAE attraverso quattro diversi linker dipeptidici. I prodotti contenenti un legame valina-citrullina o valina-alanina hanno dimostrato una alta stabilità in siero e una superiore attività in vivo, in confronto a quei coniugati basati su ponti valina-arginina o valina-lisina. Tuttavia, in nessuno dei casi abbiamo osservato una completa cura. Per investigare l’importanza dell’affinità del ligando per CAIX, abbiamo coniugato vedotina (valina-citrullina-MMAE) ad un derivato di acetazolamide ad affinità maturata, chiamato AAZ⁺, isolato precedentemente dal nostro gruppo utilizzando la tecnologia delle librerie chimiche codificate da DNA. Il nuovo prodotto SMDC viene ben tollerato ed ha una potente attività in vivo. Cure complete e durative sono state ottenute in topi trapiantati con tumori, quando il prodotto AAZ⁺-valina-citrullina-MMAE viene combinato con L19-IL2, una proteina di fusione in fase clinica per che trasporta interleuchina-2 alla matrice tumorale extracellulare. Infine, in questo lavoro è riportato il primo confronto diretto tra prodotti SMDC ed ADC non-internalizzanti. A questo scopo, AAZ⁺-valina-citrullina-MMAE viene confrontato con un prodotto ADC anti-CAIX chimicamente definito. Entrambi i prodotti dimostrano una potente attività anti-tumorale in un modello tumorale murino. In contrasto con il prodotto
SMDC, non abbiamo potuto dimostrare in modo univoco che l’attività del prodotto ADC deriva esclusivamente dalla sua selettività per CAIX. Infatti, quando l’anticorpo anti-CAIX è stato rimpiazzato da un anticorpo non specifico per il target come controllo negativo, abbiamo osservato una evidente attività anti-tumorale, il che suggerisce che (almeno in parte) l’effetto terapeutico degli ADC potrebbe essere dovuto ad un lento rilascio in circolo di potenti payload citotossici.

Il quadro dell’attività svolta potrebbe avere importanza clinica, dato che anidrasi carbonica IX è sovra-espressa nella maggioranza di carcinomi a cellule renali (RCC) e in tumori in ipossia. I prodotti SMDC basati su AAZ e AAZ⁺ rappresentano una promettente classe di prodotti biofarmaceutici per il trattamento di tumori CAIX-positivi. Inoltre, i nostri risultati danno un forte razionale per l’utilizzo di farmaci immunostimolatori, ad esempio proteine di fusione contenenti interleuchina-2, come agenti di combinazione per coniugati piccola molecola-farmac.
II. INTRODUCTION

Cancer remains a major health problem worldwide, despite advances in oncology [1]. 14.1 million new cases and 8.2 million cancer deaths occurred in 2012 worldwide. Lung cancer remains the most common type of malignancy with 1.8 million cases and 1.6 million deaths, followed in terms of incidence by breast cancer (1.7 million cases), colorectal cancer (1.4 million cases), prostate cancer (1.1 million cases) and liver cancer (782,000 cases). These numbers represent 55% of the global incidence burden in 2012 [1].

The traditional approach based on surgery, radiation and administration of small-molecule cytotoxic drugs represents the backbone of the modern way to treat most of cancer malignancies. Nevertheless, the scientific research is continuously making efforts to find more effective therapies against cancer. For example, antibody-based drugs that boost the activity of the host immune system, like the immune checkpoint inhibitors Nivolumab, Pembrolizumab and Ipilimumab, are rapidly gaining importance [2-4]. The FDA approval of Kymriah, a CAR-T cell product, as the first cell-based therapy for cancer, is attracting considerable scientific, industrial and medical interest [5, 6]. Antibody-cytokine fusion proteins [7] and targeted cytotoxics [8-10] are also considered promising therapeutics to overcome the limitations of conventional cancer therapy.

This thesis is focused on the development of non-internalizing antibody- and small molecule-drug conjugates for the treatment of solid tumors, as therapeutic alternative to conventional cytotoxics and small molecule-therapeutic agents.

1. Conventional Cancer Chemotherapy

The treatment of patients with various forms of cancer (and especially those with disseminated disease) often involves the administration of cytotoxic drugs, which are used to limit the uncontrolled tumor growth and, ideally, induce a remission [12]. Most anti-cancer drugs inhibit cell viability and/or proliferation by impacting on essential biochemical processes, such as DNA replication, transcription or protein synthesis. Some agents bind to nucleic acids or alkylate them. Tubulin binders interfere with cell division. For hormonal sensible tumors, inhibitors of hormone function may also be considered [12, 13]. Figure II.1 represents a timeline of the key events in the development of conventional cancer chemotherapy.

**Figure II.1** Development of conventional cancer chemotherapy: timeline. Adapted from ref. [14].
It is generally assumed that anti-cancer drugs act directly on tumor cells, but the interaction with endothelial cells [15] or with certain leukocytes (e.g., regulatory T cells) could also contribute to the therapeutic effect. While chemotherapy can induce long-lasting complete remissions in previously incurable disseminated malignancies (e.g., testicular cancer and certain hematological cancer types) [16, 17], the majority of metastatic forms of cancer cannot be cured by cytotoxic agents [18-21].

One of the main limitations of conventional cancer chemotherapy relates to the inability of small organic drugs to reach tumor cells. Nuclear Medicine studies, performed in patients using radiolabeled preparations of anti-cancer agents, have shown that only a small portion of the drug reaches the neoplastic masses in vivo [22] (figure I.2). These findings are consistent with the results of many quantitative biodistribution investigations, performed in tumor-bearing rodents, which indicate a limited ability of most conventional anti-cancer drugs to preferentially localize at the site of disease [9].

**Figure I.2** Positron Emission Tomography imaging of a mesothelioma patient, who received $^{11}$C-labeled docetaxel. At no time point the neoplastic lesions were visible, while a substantial accumulation of the drug in other organs could be observed. Adapted from ref. [22].
2. Targeted Delivery of Cytotoxic Drugs

An effective cancer treatments requires: (i) early diagnosis of the disease, (ii) precise localization of the lesions, (iii) maximization of cancer cell killing process and (iv) minimization of side effects. A selective drug delivery of cytotoxic agents has the potential to address all these four issues and aims at a preferential release of the toxic cargo at the site of disease, sparing normal organs [23]. Over the past twenty and more years this research area has been extensively investigated and several active (i.e. antibodies or small ligands) [8-10, 24, 25] and passive (polymers) [26] pharmacodelivery vehicles have been proposed and characterized. This chapter is solely focused on active ligand-based drug delivery strategies (i.e., on antibody-drug conjugates, ADCs, and small molecule-drug conjugates, SMDCs; figure I.3).

![Antibody-Drug Conjugate (ADC) and Small Molecule-Drug Conjugate (SMDC)](image)

**Figure I.3** Schematic representation of the basic elements in antibody-drug conjugates and small molecule-drug conjugates. Between the three fundamental modules (targeting moiety, linker and drug), spacers can be included to modify physicochemical properties of the conjugate, or to module the rate of drug release.

### 2.1 Antibody-Drug Conjugates

Three main driving forces have prompted researchers to use antibodies as vehicles for drug delivery: (i) the exquisite specificity by which they can recognize target antigens; (ii) the fact that monoclonal antibodies can be raised against virtually all proteins of interest; (iii) previous experience with radiolabeled antibody products, which aimed at concentrating therapeutic radionuclides at the tumor site. Cytotoxic drugs represent a convenient class of non-radioactive anti-cancer payloads.
ADC products result from the conjugation of a cytotoxic agent and a monoclonal antibody (mAb), using a suitable linker [25, 27]. Most of the antibodies that have been used for ADC development display insufficient anti-tumor activity, when administered as “naked” immunoglobulins [28-30]. On the other hand, the role of the mAb moiety in ADC products mainly consists in the selective delivery of a cytotoxic compound at the tumor site, where the latter is released and acts on cellular targets causing direct damage. According to this mechanism of action, ADCs can be considered as pro-drugs and the release of the cargo is of fundamental importance for therapeutic activity.

After the first wave of ADC products, based on low-potency payloads (e.g., doxorubicin, methotrexate derivatives), researchers rapidly realized that only ultra-potent drugs would allow the generation of ADCs, which can be administered at reasonably low dose and with acceptable cost-of-goods [10, 25]. An alternative strategy, which is still being pursued by a number of companies (e.g., Mersana), makes use of medium-potency anti-cancer drugs, which are attached in numerous copies to the antibody molecule, by means of multivalent (e.g., dendrimeric) linkers.

In general, ADCs are developed on the basis of tumor-targeting antibodies, directed against an antigen on the surface of the tumor cell, which rapidly internalizes, thus acting as “Trojan horse” for the delivery and subsequent release of a cytotoxic payload inside the cell. The strict requirement for ADC internalization has recently been challenged. Evidence of potent activity in preclinical models of cancer has been reported for non-internalizing ADC products, directed against a number of targets, including collagen IV, fibrin, splice isoforms of fibronectin and of tenascin-C [31-34].

The therapeutic activity and selectivity of ADC products in preclinical models of cancer can be sensational. For example, brentuximab vedotin (a product marketed under the trade name of Adcetris™) cured tumor-bearing mice at doses as low as 1 mg/Kg, corresponding to 1/30th of the
maximal tolerated dose [35]. Unfortunately, the therapeutic window of ADC products appears to be less wide in patients, for reasons which are still not completely understood. It is possible that the tumor:organ ratios observed with ADC products in tumor-bearing mice are substantially better, compared to the values observed in patients with cancer. Quantitative biodistribution studies in rodent models and dosimetric findings in nuclear medicine trials suggest that this may indeed be the case.

Four ADC products have recently gained marketing authorization: Adcetris™ (for the treatment of certain forms of Hodgkin’s lymphoma and Anaplastic large cell lymphoma), Kadcyla™ (for the second-line treatment of HER2-positive metastatic breast cancer), Besponsa™ (for the treatment of adults with relapsed or refractory B-cell precursor acute lymphoblastic leukemia) and Mylotarg™ (for newly diagnosed and relapsed or refractory CD33-positive acute myeloid leukemia).

The first approved ADC, Mylotarg™, is composed of gemtuzumab, an anti-CD33 antibody, which is conjugated to the cytotoxic antitumor antibiotic chalicheamicin via a bifunctional bridge (figure I.4). Despite its market withdrawal in 2010 [36, 37], this product has now been reapproved in adults with newly diagnosed CD33-positive acute myeloid leukemia (AML), and adults and children 2 years and older with relapsed or refractory CD33-positive AML [38].
Figure I.4 Schematic representation of the structure of Mylotarg™.

Adcetris™ (figure I.5), an anti-CD30 ADC, was approved in 2011 for the treatment of CD30-positive Hodgkin lymphoma and systemic anaplastic large cell lymphoma on the basis of the high proportion of objective responses, observed in patients, who did not have a therapeutic alternative [39]. These initial findings have later been confirmed in a double-blind Phase III clinical trial, administering the product as consolidation therapy after autologous stem-cell transplantation in patients with Hodgkin’s lymphoma at risk of relapse or progression [40].

Kadcyla™ is composed of the anti-HER-2 humanized antibody trastuzumab, coupled to the potent maytansinoid DM1 via a non-cleavable linker (figure I.5). It is believed that the payload is released inside the tumor cells, after ADC internalization and subsequent proteolytic digestion of the antibody moiety in an endo-lysosomal compartment [9]. In 2013, Kadcyla™ received FDA approval for the treatment of patients with HER2-positive metastatic breast cancer who had previously received trastuzumab and a taxane, separately or in combination [9]. However, the product has recently failed to demonstrate
superiority over trastuzumab in combination with standard chemotherapy in a Phase III clinical trial in first-line patients.

Figure I.5 Schematic representation of the structure of Adcetris™ and Kadcyla™. DAR: drug-antibody ratio.

Besponsa™ (inotuzumab ozogamicin) has been approved in 2017 as monotherapy for the treatment of acute lymphoblastic leukemia (ALL), which is an uncommon disorder affecting about 20% of adults with acute leukemia [41]. The approval is based on the results of INO-VATE, a multicenter phase III clinical trial evaluating the safety and efficacy of the product in 326 relapsed or refractory ALL patients. Besponsa™ consists of the humanized anti-CD22 monoclonal antibody inotuzumab, which is linked to ozogamicin via a hydrazine-disulfide linker.

The majority of ADC products, which have been investigated in the clinic, exhibited certain levels of bone marrow toxicity, even though the cognate antigen was not expressed in that compartment. It is likely that premature drug release may contribute to these side effects. In addition, complete responses have been reported in patients treated with Adcetris™, who did not exhibit a detectable expression of the cognate CD30 antigen [42].
It is not clear, at this moment in time, whether the observation of ADC activity in antigen-negative patients may be due to technical problems in the execution of immunohistochemical experiments or to an intrinsic property of the antibody-drug conjugate, which circulates in blood for several days and slowly releases its cytotoxic payload.

2.2 Small Molecule-Drug Conjugates

Antibodies in IgG format exhibit a long circulatory half-life (mediated by interaction with the neonatal Fc receptor FcRn) and extravasate slowly. As a result, acceptably high tumor:organ ratios are observed in vivo only several days after intravenous administration [43]. The limited diffusion properties of monoclonal antibodies were illustrated by Dennis and coworkers, who investigated the microscopic accumulation of Trastuzumab in mice bearing HER2-positive tumors [44]. Immunofluorescence detection studies revealed the inability of the mAb in IgG format to penetrate the tumor mass, with a striking accumulation on perivascular tumor cells (“antigen barrier effect”), failing to reach the majority of neoplastic cells (figure I.6).

Figure I.6 (A) Macroscopic distribution of a radiolabeled preparation of the anti-HER2 antibody trastuzumab ($^{64}$Cu-DOTA-trastuzumab) in a breast cancer patient, at different time points (1, 24 and 48 hours) following intravenous injection. The antibody remains for hours in circulation and reaches efficiently the liver (excretory organ of full IgG molecules), missing selective tumor accumulation. Adapted from ref. [45]. (B) Microscopic images of a tumor tissue after intravenous administration of fluorescently labeled trastuzumab (green). The vast majority of antibody molecules localizes around blood vessels (stained in red) and does not reach the deep tumor mass (blue). Adapted from ref. [44].
The suboptimal biodistribution properties of immunoglobulins have prompted researchers to use small organic ligands as alternative vehicles for drug delivery applications. Unlike antibodies, small organic molecules can get out of blood vessels in a matter of seconds (Figure 1.7) [46].

![Figure 1.7 Intravital imaging of the distribution of a small drug (a PARP inhibitor) in a murine model of cancer. Few seconds after intravenous administration, the drug (green signal) perfuses the tumour via its functional vessels (central picture) and extravasates within minutes (right picture). The compound distributes non-specifically within tumore cells (red). Adapted from ref. [46].](image)

In the absence of a suitable binding event (e.g., recognition of a membrane antigen or of an extracellular matrix component), small molecules rapidly diffuse back into blood and can be excreted. However, small ligands with a sufficiently high binding affinity to accessible tumor-associated antigens can exhibit excellent tumor:blood and tumor:organ ratios, combining a rapid extravasation, a rapid elimination and a long residence time at the site of disease [8, 9]. Suitable ligands for drug delivery applications include folate analogues [8, 47], prostate-specific membrane antigen (PSMA) ligands [48] and binders to carbonic anhydrase IX (CAIX) [49].

A folate-vinblastin conjugate named Vintafolide (figure 1.8), developed by Endocyte, exhibited encouraging clinical results for the treatment of advanced ovarian cancer, especially in patients with high uptake of a radiolabeled folate derivative used as companion diagnostic [50]. These data led to a conditional approval in Europe, but product development
activities were later discontinued on the basis of disappointing Phase III trial results.

![Vintafolide](image)

**Figure 1.8** Chemical structure of Vintafolide. The folic acid moiety (in red) serves as high-affinity binding ligand for the folate receptor ($K_D \approx 0.1$ nM). A vinca alkaloid cytotoxic molecule (blue) is covalently attached to the targeting moiety through a reducible linker and a peptide spacer.

Encouraging preclinical results with PSMA ligand derivatives [48, 51, 52] have motivated Endocyte to develop an SMDC product for the treatment of advanced prostate cancer. The drug conjugate EC1169 makes use of a very potent tubulysin payload (figure I.9) and is currently investigated in a Phase I clinical trial, together with a companion diagnostic product.
Therapeutic radionuclides have been considered as an alternative avenue for arming PSMA ligands. A recent clinical report on 148 patients with metastatic castration-resistant prostate cancer, treated with the $^{177}$Lu-labeled-PSMA-617 radiopharmaceutical (figure I.9), evidenced favorable safety and excellent efficacy, with >40% of subjects enjoying an objective biochemical and radiological response [53]. Recent results on PSMA-targeted radiotherapy indicate that the use of α-emitting radionuclide payloads (e.g., $^{225}$Ac) may be superior to the β-emitting $^{177}$Lu (figure I.10) [54, 55].
Figure I.10 $^{68}$Ga-PSMA-11 PET/CT scan of a patient with metastatic prostate cancer (A). While the initial treatment with the $\beta$-emitter $^{177}$Lu-PSMA-617 was not efficacious (B), restaging after multiple administration of the $\alpha$-emitter $^{225}$Ac-PSMA-617 (C and D) showed impressive response. Serum prostate-specific antigen (PSA) levels confirmed the radiological exam. A similar response to $^{225}$Ac-PSMA-617 was observed in a second patient (data not shown) not eligible to be treated with $\beta$-emitter radiopharmaceuticals. Adapted from ref. [55].

Other targets for the development of SMDC products include somatostatin receptors, the aminopeptidase CD13 and certain integrins.

Unlike antibodies, which can be raised against virtually any antigen, small molecule protein ligands can be difficult to generate, depending on the nature of the cognate target. Nowadays, fully human monoclonal antibodies can be rapidly isolated from large combinatorial phage display libraries, containing billions of different specificities [56]. The advent of DNA-encoded combinatorial chemical libraries allows the creation and screening of compound collections of unprecedented size [57-62], thus facilitating ligand discovery. These libraries are composed of small organic molecules, individually attached to distinctive DNA fragments serving as amplifiable identification barcodes. Members of DNA-encoded combinatorial libraries can be screened simultaneously by selection with the target protein of interest, immobilized on a solid support. Preferential binders are identified by high-throughput DNA sequencing techniques.
The role that DNA-encoded chemical library technology can play for the development of SMDC products is illustrated in the following example. Mice bearing subcutaneously-grafted renal cell carcinoma xenografts were injected with a near-infrared fluorescent dye or with dye derivatives, coupled to acetazolamide or to a high affinity acetazolamide derivative ($K_D = 200$ pM), isolated from a DNA-encoded chemical library [63] (figure I.11). Fluorescence imaging procedures revealed that the unconjugated dye was not able to localize to the tumor and was rapidly excreted, in full analogy to the behavior of standard chemotherapeutic drugs, illustrated in figure I.2. On the other hand, the dye conjugate with acetazolamide revealed a rapid and selective tumor targeting, which was visible already 1 hour after intravenous administration. The intermediate affinity of the conjugate towards CAIX ($K_D = 8$ nM) did not allow a long residence time on the neoplastic lesion. By contrast, the higher affinity CAIX targeting agent ($AAZ^+$), exhibited a rapid, long-lasting and selective accumulation in the tumor mass. Similar results were obtained using quantitative biodistribution studies, using radiolabeled preparations of the same ligands [9, 64, 65].

**Figure I.11** Near-infrared fluorescence imaging of tumor-bearing mice, imaged at various time points following intravenous injection of a dye, or of the same dye coupled to two different CAIX ligands, differing in binding affinity to the target. Ligand 1 = acetazolamide. Ligand 2 = $AAZ^+$. Adapted from ref. [63].
3. Carbonic anhydrase IX as target for cancer therapy

3.1 Carbonic Anhydrase IX Expression in Solid Tumors

Carbonic anhydrases are metalloenzymes that can be found in most living organisms, where they catalyze the hydration of carbon dioxide to bicarbonate. Fifteen human isoforms that differ in tissue expression patterns, kinetic properties and subcellular localization are currently known [66]. Their physiological roles are typically related to pH regulation and with the active transport of CO₂ and hydrogen carbonate through cellular membranes [67, 68]. Among all the CA isoforms, CAIX (formerly referred to as MN antigen; figure I.12) is a transmembrane homodimeric enzyme over-expressed in more than 90% of clear cell renal cell carcinoma (ccRCC) subtypes [69].

![Figure I.12 Schematic representation of CAIX. The protein is constituted by four domains: an N-terminal proteoglycan-like domain (orange), a zinc-containing extracellular catalytic domain (grey), a trans-membrane region and a short intracellular tail. The protein tends to form homodimers in its native form. Adapted from ref. [70].](image)

The high expression level of CAIX in ccRCC is due to a mutation in the von Hippel-Lindau (VHL) tumour suppressor gene, which normally acts as negative regulator for the expression of the protein [71]. In addition, CAIX is one of the best markers of hypoxia and, as such, can be found in many tumor types, especially those characterized by a low oxygen...
concentration [72]. The pattern of expression of CAIX in healthy organs is limited on the first portion of the gastro-intestinal tract (e.g., stomach, duodenum and gallbladder; figure I.13) [73].

Figure I.13 CAIX expression pattern in human healthy organs (black outline) or cancer tissues derived from patients (red outline). The antigen, stained in black by immunohistochemistry, is selectively expressed in the first portion of the gastrointestinal tract (i.e., stomach and small intestine) and in different malignancies. Pictures were taken from randomly selected samples published in the human protein atlas (www.proteinatlas.org).
3.2 CAIX Inhibition as a Therapeutic Anti-Tumor Strategy

Carbonic anhydrases inhibitors (CAIs) have been clinically exploited as systemic diuretics and anti-glaucoma agents [74, 75]. The application of small molecules inhibitors of CAIX as anti-cancer agents has shown to retard tumor growth [76], inhibit metastasis [77] and cancer stem cell expansion in preclinical models of breast cancer [78]. Our group previously reported the modest anti-tumor activity of acetazolamide, a low-nanomolar binder of CAIX, in SKRC-52 renal cell carcinoma bearing mice [79]. These results have not yet been translated to patients. Classic CAIs are represented by primary sulfonamides (RSO₂NH₂) or sulfamates (RSO₃NH₂) and around 30 clinically used drugs belong to this category [80]. Coumarins and phenols have been also proposed as carbonic anhydrase ligands [81]. While binding affinities are in general very high (K_D values are typically below 100 nM), a good selectivity towards the different CA isoforms is still hard to obtain. However, the generation of charged aromatic sulfonamides limits penetration through the cell membrane and restricts binding to cell-surface CA isoforms.
3.3 Carbonic Anhydrase IX as a Target for Drug Delivery

The selective expression pattern of CAIX in malignancies versus healthy organs stimulated its clinical investigation as target for the delivery of radionuclides (figure I.14) [82, 83] and of cytotoxic agents [84].

![Figure I.14](image)

**Figure I.14** Body scan (anterior and posterior) of a metastatic RCC patient that received a radiolabeled preparation of Girentuximab (I31-cG250). Adapted from ref. [85]. Previously undetected metastases were visualized. Similarly to this example, other patients with confirmed antigen-positive tumors (n = 13), showed selective tumor uptake of the radioactive antibody.

Although in the early development anti-CAIX therapeutics were designed to be internalized by tumor cells, our group recently reported the inefficient internalization of the protein upon ligand binding [49]. Petrul and coworkers explored the conjugation of an anti-CAIX mAb to MMAE, through the cleavable valine-citrulline linker, to generate the ADC BAY 79-4620 [84]. The group demonstrated the selective affinity of this product to the CAIX isoform and its ability to selectively kill CAIX-positive cancer cells in vitro, by tubulin disruption. BAY 79-4620 was also shown to be effective in vivo in mice grafted with HT-29 and Colo205 colorectal
tumors or with cervix carcinoma HeLa-MaTu tumors, at doses between 5 and 10 mg/kg. A modest anti-cancer activity was reported against other cancer models, albeit at higher doses (30-60 mg/kg). However, while free MMAE (0.2 mg/kg, equivalent to 10 mg/kg of ADC) was less effective than BAY 79-4620, the efficacy of paclitaxel administered at the dose of 15 mg/kg was comparable to the one described for the ADC. In 2014, BAY 79-4620 entered a phase I dose-escalation clinical study with 12 patients, bearing histologically or cytologically confirmed solid tumors [86]. The product was administered at doses ranging from 0.3 to 4.6 mg/kg. While no complete or partial response were reported, treatment-related side effect occurred in the majority of patients and the highest dose led to patient death due to cardiac arrest and pancreatitis. This tragic event underlined the importance of an accurate preliminary evaluation of the antigen expression in patients, since data of CAIX expression in the studied tumors were available for only 50% of patients, among whom only 2 showed more than 10% antigen-positive cells in the tumor mass.

Our group has previously described a series of SMDC products, featuring the low-nanomolar CAIX binder acetazolamide as tumor-homing moiety. DM1, a potent anti-tubulin drug also present in Kadcyla™ has been coupled to acetazolamide using a reducible disulfide bond as a linker structure cleavable in the extracellular space (figure I.15). The corresponding products exhibited a promising anti-cancer activity [49, 87], which was superior to the one of sunitinib and sorafenib (two standard drugs for the treatment of metastatic kidney cancer) in tumor-bearing mice. As for SMDCs targeting the folate receptor or PSMA, the tumor targeting performance of CAIX ligands can be monitored using $^{99m}$Tc-labeled companion diagnostics [88] (figure I.15).
Figure I.15 Molecular structure of AAZ-SS-DM1, a CAIX-targeted SMDC, and of PHC-102, a CAIX-targeted imaging agent. In AAZ-SS-DM1 acetazolamide (red), a nanomolar binder of CAIX, is covalently attached to a maytansinoid potent derivative (DM1, blue) by a disulfide reducible bridge and a peptide spacer. In PHC-102 the same targeting moiety acetazolamide is radiolabeled with technetium-99m (blue).

4. Internalizing versus Non-Internalizing Targeted Cytotoxics

Until recently, most *active* delivery strategies of cytotoxic drugs have focused on the use of monoclonal antibodies or small organic ligands, capable of selective binding and internalization into the target tumor cells [8-10]. It has commonly been assumed that the tumor homing moiety (antibody or small organic ligand) should preferably be directed against tumor-associated antigens expressed on the surface of cancer cells. Ideally, the pro-drug would internalize upon binding to its cognate target, thus facilitating the delivery and release of the cytotoxic cargo inside the malignant cell [24, 25, 27, 89] (figure I.16).
Figure I.16 Schematic representation of possible mechanism for the drug release and cytotoxic activity of internalizing targeted cytotoxics. The conjugate, after extravasation, will diffuse through the tumor microenvironment (ECM: extracellular matrix) and meet its cognate antigen on the tumor cell membrane. If an efficient internalization process occurs, the targeted cytotoxic accumulates in the intracellular space. Once inside the cells, the linker can be cleaved and the drug payload is released in its active form, to act on the intracellular target causing localized cancer cell death.

However, in principle, it would be conceivable to develop non-internalizing ADC and SMDC products, which liberate their toxic payload in the extracellular environment [90] and the availability of novel antibodies, with exquisite tumor-targeting properties, has prompted the investigation of ADC products based on non-internalizing ligands. In this case, the linker cleavage and the subsequent drug release should preferably take place in the tumor microenvironment, allowing the subsequent diffusion of the active payload and its internalization into neighboring neoplastic cells. Since passive diffusion is a non-specific process, the cytotoxic agent has the potential to reach antigen-negative cancerous cells within the tumor mass (a mechanism often referred to as "bystander effect"). In principle, non-internalizing ADCs and SMDCs may display potent therapeutic activity against tumors with high mutation rates or characterized by antigen loss, where certain cell populations can develop resistance to conventional internalizing targeted cytotoxics. Potentially, the bystander killing effect could also impair structures which support tumor growth, such as stromal cells, leukocytes and tumor blood...
vessels, thus enhancing the anti-tumor effect of the product [91] (figure I.17).

Figure I.17 Schematic representation of possible mechanisms for the drug release and cytotoxic activity of non-internalizing targeted cytotoxics. The antigen is represented as an element of the tumor microenvironment (ECM: extracellular matrix). After extravasation the pro-drug will bind the tumor-associated antigen. A linker cleavage event liberate the drug payload that will diffuse through cellular membrane of cancer cells or of other stromal structures (like endothelial cells) and act on cognate intracellular target. In the last step dying cells release a large number of cleaving agents like enzymes and reducing compounds to start a self-amplified cleavage cascade. The same series of events can in principle occur when the tumor-associated antigen is expressed on the surface of cancer cells, and, upon binding, the drug-conjugate is not efficiently internalized.

In the case of non-internalizing SMDC products, the cleavage of the linker in the extracellular matrix and the diffusion of the drug through the cancer cell membrane may happen in concert with a preferential accumulation of the pro-drug at the tumor site, thus leading to a selective anti-neoplastic action. Some non-internalizing SMDC products have shown, indeed, very potent anti-tumor activities in preclinical models of cancer. For example, anti-CAIX SMDC products represent a promising example of non-internalizing pharmaceutical agents in industrial development [49].
5. Cytotoxic Payloads and Linker Technologies

Together with the tumor-homing moiety development, the design of a proper linker and the choice of a suitable payload are crucially important for the generation of efficacious and well-tolerated ADCs and SMDCs.

5.1 Linkers

A list including common linker structures applied for the development of targeted cytotoxics is reported in **table II.1**.

<table>
<thead>
<tr>
<th>Linkage</th>
<th>Structure</th>
<th>Cleavage Mechanism</th>
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<tbody>
<tr>
<td>Disulfide</td>
<td><img src="image" alt="Disulfide Structure" /></td>
<td>Reduction</td>
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<tr>
<td>Amide</td>
<td><img src="image" alt="Amide Structure" /></td>
<td>Hydrolysis (e.g. proteolysis)</td>
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<td>Carbamate</td>
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<td>Hydrolysis</td>
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<tr>
<td>Hydrazone</td>
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<td>Hydrolysis</td>
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<tr>
<td>Ester</td>
<td><img src="image" alt="Ester Structure" /></td>
<td>Hydrolysis</td>
</tr>
</tbody>
</table>

**Table II.1**

A main requirement to prevent premature drug release and related side effects from ADC and SMDC products is the high stability of the linker in plasma, after administration. Provided that a sufficient amount of drug conjugate reaches intact the tumor, a second key attribute of the linker relates to its ability to efficiently release the payload at the tumor site. This section is focused on reducible bonds or on protease-cleavable peptides, as suitable linkers for the generation of internalizing or non-internalizing ADCs and SMDCs.
Glutathione (GSH) represents the most abundant thiol and reducing agent in the intracellular space, both in normal cells and in tumors, which often contain higher concentrations of this species [92, 93]. While disulfide-based linkers have been designed for the intracellular release of anti-cancer drugs, the same chemical structures can be considered for the extracellular drug release, as a consequence of tumor cell death and increased GSH concentration. Disulfides are typically stable in the absence of free thiols at physiological pH, with a serum half-life that can be longer than one week. In vivo, certain disulfide-based ADCs have exhibited stability in blood for 2-4 days. Moreover, this stability can be dramatically improved by increasing the steric hindrance of substituents at the cleavage site [94, 95]. This type of linkage can be established, in the easiest case, between thiol residues of the toxic drug and a cysteine present on the delivery vehicle (e.g. a mAb) [96], thus generating a traceless cleavable disulfide bridge (figure I.18). When the drug does not contain thiol groups, or the modification with an adaptor is not tolerated in terms of activity, the use of a self-immolative linker may be considered (figure I.18) [48, 97].

Figure I.18 Disulfide-based linker structures. Red = tumor homing ligand. Blue = drug payload. a) Traceless disulfide linkers; b) disulfide linkage suitable for ligand conjugation to amine groups of the drug payload; c) different combinations of disulfide bonds and self-immolative linkers.

Most of the products bearing disulfide linkers [8-10, 98] have been designed to release the payload through receptor-mediated endocytosis.
However, it is conceivable to assume that the tumor environment *in vivo* may be more complex, with dying cells constantly releasing reducing agents to the surrounding areas. Indeed, non-internalizing ADC and SMDC products based on disulfide bonds can potentially be cleaved in the tumor extracellular milieu, releasing a payload that may diffuse and be subsequently taken up by neoplastic cells. The release of GSH to the extracellular environment may generate a self-amplifying cycle of cell death, glutathione release and subsequent pro-drug activation.

As an alternative to disulfides, certain peptide sequences have been used as linkers for the generation of ADC and SMDC products (figure I.19). These functional groups combine a high systemic stability with a rapid release of the drug at the site of disease. Indeed, proteolytic enzymes, such as cathepsin B, urokinase-type plasminogen activator and matrix metalloproteinases (MMPs) that are involved in cancer progression features like angiogenesis, invasion and metastasis [99], may be over-expressed both at the tumor site, both in intra- and extracellular compartments [100]. Similarly to the cleavage of disulfide bonds, proteolytically-cleavable linkers could be exploited also for the release of drugs in the extracellular tumor microenvironment. Indeed, the protease-mediated release of payloads from non-internalizing ADCs and SMDCs can be amplified by tumor cell death, which sheds a large number of proteolytic activities into the cancer microenvironment. This line of research led to the use of a valine-citrulline containing linker in internalizing products like the marketed Adcetris™ and in other clinical-stage candidates [101, 102]. This dipeptide structure, historically proposed as a specific substrate of the lysosomal cysteine protease cathepsin B and therefore associated with internalizing ADC development, has been exploited for the generation of non-internalizing products [34, 103, 104]. Recent scientific reports indicate that the valine-citrulline linker mediates drug release upon proteolytic cleavage in the presence of other proteases, like the extracellular serine hydrolase Carboxylesterase 1C (Ces1C) [105], and that the expression of cathepsin B is dispensable for the *in vivo* anti-cancer activity of valine-citrulline
based ADC products [106]. In particular, researchers at Genentech demonstrated that suppression of cathepsin B expression via CRISPR-Cas9 gene deletion did not hinder the \textit{in vivo} efficacy of valine-citrulline containing ADC products. In this example, mass spectrometry experiments suggested that the presence of other extracellular proteases (like cathepsin K, L and S) might be relevant for the \textit{in vivo} activation of the ADC pro-drugs [106].

![Proteolytic cleavage](image)

**Figure 1.19** Peptide-based linker structures and mechanism of drug release. Red = tumor homing ligand. Blue = drug payload.

The use of non-cleavable linkers may represent an alternative to cleavable linkers (e.g., those based on disulfide bonds or peptides) and is considered to be particularly suitable for those products that undergo an efficient internalization. For example, non-cleavable amide bonds have been used for the coupling of different classes of cytotoxic agents (e.g. maytansinoids [107], calicheamicins [108], cryptophycins [109] and auristatins [110]) to internalizing antibodies, aiming at the selective release of the toxic moiety in the intracellular space of malignant cells, exploiting the proteolytic degradation of the immunoglobulin.

**5.2 Payloads**

Even the best ligand-linker combination will be inefficacious if the attached therapeutic moiety lacks the ability to kill tumor cells, once it is released at the site of the malignancy. The \textit{in vivo} potency of the payload does not only depend on intrinsic toxicity of the free drug, but also on intra- and extra-cellular metabolism, susceptibility to multidrug efflux pumps and ability to reach the intracellular target.
Figure I.20 Structures of the most common potent cytotoxic drugs used as payload in ADC and SMDC development. Mechanism of action and growth inhibiton data as average (av.) IC50 values are given (cell lines tested are reported in brackets) [9, 111].

As only a limited number of therapeutic molecules are usually carried by one ligand (often one or few drug molecules are attached to one delivery vehicle in the case of SMDC products) the potency of the payload must be high enough to maximize killing of cancer cells. Furthermore, target
saturation may prevent dose escalation above certain threshold levels. Cytotoxic compounds with a low nanomolar or sub-nanomolar IC\textsubscript{50} are typically considered to be adequate and products that are currently investigated in clinical trials are based on high-potency drugs [8, 9, 27] (figure I.20).

Highly potent cytotoxic drugs used as payloads in targeted cytotoxic products include maytansinoids [112, 113], duocarmycins [114, 115], dolastatin derivatives like monomethyl auristatin E (MMAE) [116, 117], anthracyclines [104, 111, 118], tubulysins [119-123], pyrrolobenzodiazepines [124, 125] and calicheamicins [126, 127]. Notably, these drugs are too potent to be applied in chemotherapy in an unmodified form, and they typically exhibit a narrow therapeutic window \textit{in vivo}.

6. Future Perspectives

ADCs and SMDCs are pro-drugs with the potential to substantially improve the therapeutic index of the corresponding cognate drug. Numerous products (especially ADCs) are currently being investigated in clinical trials, even though many companies are reducing their research and development activities in the field, after substantial initial investments. One of the main unresolved challenges, which complicates the clinical and industrial development of ligand-based targeted cytotoxics, relates to the difficulty in translating promising preclinical results (i.e., cancer cures in tumor-bearing mice) into a corresponding clinical activity. A reliable quantification of the concentration of cytotoxic agent delivered to neoplastic lesions in mouse and man will be of fundamental importance, in order to rationally advance ADC and SMDC research.

Nuclear Medicine imaging studies will be extremely useful, in order to quantify product uptake. In particular, it will be important to learn whether lesions that benefit most from pharmacological treatment are also the ones with the highest product uptake in the neoplastic mass and in
normal organs. At present, these studies are hindered both by the complexity of coordinating Oncology and Nuclear Medicine investigations in the same patient and by regulatory aspects (e.g., GMP preparations for both the unlabeled and labeled products).

Until now, clinical-stage ADC and SMDC products have rarely demonstrated an ability to induce cancer cures in patients, when used as monotherapy. The opportunity to combine targeted cytotoxics with certain immunostimulatory drugs is becoming increasingly attractive, also in view of the growing role played by immune-oncology drugs for the treatment of patients with metastatic disease. The antibody-based delivery of interleukin-2 (IL2, a potent activator of T cells and NK cells) has been shown to potentiate chemotherapy in preclinical models of cancer, especially when administered after the cytotoxic agent [128]. It is likely that the tumor becomes more immunogenic, after the insult caused by the action of cytotoxics. Possible mechanisms may involve the up-regulation of stress surface proteins on tumor cells (e.g., MIC-A) and the subsequent recognition by NK cells, or the increased presentation of tumor-associated peptides on MHC class I molecules. This hypothetic mechanism of synergy, when clinically confirmed, may pave the way for the use of targeted cytotoxics (SMDCs and ADCs) in combination with immunocytokines, as a new strategy to treat cancer.

Looking into the future, we anticipate that ADCs and SMDCs may play an increasingly important role not only in Oncology, but also for the delivery of other classes of drugs (e.g., antibiotics, anti-inflammatory drugs) [129]. In our opinion, the therapeutic potential of ligands coupled to small organic drugs will be best exploited in combination with innovative immunomodulatory modalities, such as immunological check-point inhibitors or antibody-cytokine fusion proteins [130, 131]. Finding the right combination partners will require substantial research efforts, but may pave the way to therapeutic modalities with unprecedented potency and selectivity.
7. Aim of the Thesis

The aim of this thesis was to generate a new series of non-internalizing drug conjugates targeted against the cell surface tumor-associated antigen carbonic anhydrase IX (CAIX). Cytotoxic drugs that are commonly considered for internalizing ADC products were coupled to acetazolamide, a reversible low-nanomolar binder of the protein, through peptide cleavable linkers. The novel acetazolamide-drug conjugates were characterized for their purity, stability, in vitro and in vivo preclinical biological activity.

After having demonstrated the anti-cancer potential of a new set of SMDCs in a mouse model of renal cell carcinoma, we focused on (i) the use of an acetazolamide derivative with an improved tumor targeting performance (which was named AAZ⁺) as tumor-targeting moiety and on (ii) the combination of the new SMDC product with the clinical-stage fusion protein L19-IL2 (capable of delivering interleukin-2 to the tumor neo-vasculature). Finally, we challenged our most potent anti-CAIX SMDC in a head-to-head comparison with a novel ADC product that targets the same antigen and bears an identical linker-payload combination.

Chapter III corresponds to our paper “Acetazolamide serves as selective delivery vehicle for dipeptide-linked drugs to renal cell carcinoma”, published in Molecular Cancer Therapeutics in 2016, and describes the generation of new SMDC products based on the two potent drug payloads monomethyl auristatin E (an anti-tubulin agent derived from dolastatin-10) and PNU-159682 (a potent derivative of nemorubicin), which were coupled to acetazolamide through a valine-citrulline cleavable peptide linker.

we describe how the anti-cancer activity of non-internalizing SMDCs is influenced by the stability of the linker structure between the tumor targeting agent and the toxic payload.

Chapter V corresponds to our manuscript “Enhanced therapeutic activity of non-internalizing Small Molecule-Drug Conjugates targeting carbonic anhydrase IX in combination with targeted interleukin-2”, accepted for publication on Clinical Cancer Research. In this chapter we describe the generation of a new SMDC product based on AAZ+, a high-affinity ligand specific to carbonic anhydrase IX. Here we also describe our effort in improving the preclinical therapeutic efficacy of this novel product by combination with targeted IL2.

Chapter VI corresponds to our manuscript “Chemically-defined antibody- and small molecule-drug conjugates for in vivo tumor targeting applications: a comparative analysis”, published in Journal of American Chemical Society in 2018. In this chapter we present the first head-to-head comparison of chemically-defined non-internalizing ADC and SMDC products.

Chapter VII contains the discussion of the results of this thesis, an outlook for the next steps of our research program as well as considerations on its potential for clinical development of the products presented here.

Chapters VIII, IX, X and XI correspond to the materials and methods of the research work presented in chapters III, IV, V and VI. References, acknowledgments and my curriculum vitae follow.
III. ACETAZOLAMIDE SERVES AS SELECTIVE DELIVERY VEHICLE FOR DIPEPTIDE-LINKED DRUGS TO RENAL CELL CARCINOMA

This chapter corresponds to the paper “Acetazolamide serves as selective delivery vehicle for dipeptide-linked drugs to renal cell carcinoma.” by S. Cazzamalli, A. Dal Corso & Dario Neri published in Molecular Cancer Therapeutics, 2016, 15, 2926-35 [132]. Copyright American Association for Cancer Research. Reproduced with permission.

1. Introduction

The majority of cytotoxic agents used for the pharmacotherapy of cancer do not preferentially accumulate at the tumor site, leading to potential toxicities and to suboptimal therapeutic efficacy [8, 9, 22, 133]. In tumor-bearing mice, unfavorable tumor:organ ratios have been reported for many drugs, including doxorubicin [134], paclitaxel [135], cisplatin [136], cyclophosphamide [137], sunitinib [138], and different fluorinated pyrimidines [139], to name just a few. For example, the relative uptake of doxorubicin in the tumor (expressed as percentage injected dose per gram, or %ID/g) was found to be only 5-10% of the corresponding values in lung, heart or liver, in the time period 0.5 – 8 hours [134]. Importantly, similar trends are emerging from the positron emission tomography study of cancer patients, who had received radiolabeled preparations of cytotoxic drugs [22, 133].

The coupling of potent cytotoxic payloads to antibodies or small organic molecules, acting as selective pharmacodelivery vehicles, has been proposed as a general strategy to improve the therapeutic index of anti-cancer drugs. Indeed, certain antibody-drug conjugates (ADCs) [10, 27, 140, 141] and small molecule-drug conjugates (SMDCs) [8] have exhibited promising activity in preclinical models of cancer. Recently, two
ADC products (Adcetris™ and Kadcyla™) have gained marketing authorization for oncological applications.

Some ADCs cured tumors in mice at doses of 0.5 – 3 mg/Kg, while the same products could be administered at 60-fold greater dose with acceptable safety profiles [35]. Unfortunately, such a high therapeutic index is rarely observed in the clinic, suggesting that the tumor targeting properties of the antibody, the drug release process and/or the intrinsic sensitivity of tumor cells may be dramatically different in preclinical models and in cancer patients. Nuclear medicine studies with radiolabeled antibody preparations have previously shown that lower tumor:organ ratios are often observed in humans compared to tumor-bearing mice, possibly reflecting differences in antigen abundance and vascular permeability [142, 143]. SMDCs may represent an attractive alternative to ADC products, as smaller pharmaceuticals extravasate more rapidly and diffuse more homogenously within the neoplastic mass [8, 49, 87, 88, 144, 145]. Unlike antibodies (which can easily be raised against the majority of target proteins of interest), it is not always easy to generate high-affinity small molecule ligands to tumor-associated antigens. However, excellent tumor-targeting results have been reported for folate analogues targeting folate-receptor positive tumors [146, 147], substituted urea derivatives targeting prostate-specific membrane antigen [148], somatostatin antagonists targeting the somatostatin receptor [149] and for carbonic anhydrase IX (CAIX) ligands [49, 63, 87, 88].

It has generally been assumed that ADC and SMDC products would crucially rely on the use of ligands, capable of selective internalization of the conjugate into the tumor cells, followed by an intracellular liberation of the cytotoxic payload [10, 27, 140, 141]. This concept, however, has recently been challenged, as potent and selective anti-cancer activity has been observed with ADCs and SMDCs specific to antigens, which do not internalize [33, 49, 87, 96, 150, 151].
CAIX is a membrane protein, expressed in the majority of clear cell renal cell carcinomas [66,70,152]. CAIX is virtually undetectable in most normal adult tissues, except for certain structures in the gastrointestinal tract (stomach, duodenum, gallbladder), which are strongly positive for the antigen. Anti-CAIX monoclonal antibodies efficiently localize to metastatic renal cell carcinoma lesions in patients but, surprisingly, not to CAIX-positive stomach, duodenum and gallbladder [153], suggesting that differences in vascular permeability may account for the process.

To the best of our knowledge, CAIX is a tumor-associated target which does not internalize [49], even though at least one anti-CAIX ADC product has been studied in the clinic in patients with cancer [154]. CAIX-positive tumors can be efficiently targeted using certain small aromatic sulfonamides [49,66,70,87,88,155], including acetazolamide.

We have previously reported that acetazolamide can be used to deliver disulfide-linked derivatives of DM1 (a potent cytotoxic maytansinoid) to CAIX-positive tumors, leading to a potent anti-tumor effect [49,87]. We hypothesized that the extracellular drug release process at the tumor site would be facilitated by the death of tumor cells and the subsequent release of glutathione into the tumor extracellular space, which can work as a reducing agent on disulfide bonds, favoring an amplified cascade of drug release and tumor cell death. Since disulfide bonds can be unstable in vivo and since most anti-cancer drugs (unlike DM1) do not contain thiol groups suitable for coupling purposes, it would be interesting to learn whether potent non-internalizing SMDCs can be generated with stable peptide linkers and with other classes of drugs. In this chapter, we describe that potent anti-cancer SMDCs can be generated by coupling acetazolamide to highly cytotoxic drugs (e.g., MMAE and PNU-159682), using a linker containing a valine-citrulline moiety and a self-immolative spacer. These findings reinforce the concept that potent anti-tumor activity can be generated by the efficient release of toxic payloads in the tumor extracellular space, without ligand internalization into the target cells of interest.
2. Results

2.1 Synthesis and tumor-targeting properties of acetazolamide derivatives

Acetazolamide was coupled to a Lys-Asp-Cys-based $^{99m}$Tc-chelating moiety, which had previously been used for the imaging of cancer patients with folate derivatives [50], using a “click” chemistry reaction (figure III.1). The resulting conjugate 1 could be labeled with $^{99m}$Tc (radiolabel incorporation >95% routinely achieved; 160-210 MBq/mL) for quantitative biodistribution analysis in tumor-bearing mice. Click chemistry was also used to install the acetazolamide moiety onto Asp-Arg-Asp-Cys peptidic derivatives, which were subsequently coupled to a red Alexa fluorophore (Compound 2a), a near-infrared dye (Compound 3a) or cytotoxic drugs, based on the monomethyl auristatin E (MMAE; Compound 4a) or PNU-159682 (Compound 5a) active moieties (figure III.1). As negative controls devoid of CAIX binding, derivatives containing an amide function instead of the acetazolamide moiety were used (Compounds 2b, 4b and 5b).
Figure III.1 Chemical structures of CAIX-targeting acetazolamide derivatives. Compound 1 features a $^{99m}$Tc-chelating moiety, while compounds 2a and 3a contain respectively an Alexa594 or a IRdye680RD fluorophore moiety. Cytotoxic drug derivatives featured an acetazolamide moiety (a series) or an amide (b series, serving as negative control). Products containing the MMAE (compounds 4a and 4b) or PNU-159682 payload (compounds 5a and 5b) featured a Asp-Arg-Asp-Cys spacer, a valine-citrulline dipeptide cleavable linker, as well as a self immolative linker.

Confocal microscopy experiments were performed to investigate the internalization of our acetazolamide derivatives upon ligand binding to CAIX. SKRC-52 cancer cells (expressing high levels of CAIX) were incubated with compound 2a and the fluorescence derived from Alexa594 labeled acetazolamide was imaged after incubation for 30 and 60 min.
Compound 2a showed selective accumulation on the membrane SKRC-52 cells, as compared to CAIX-negative HEK 293 cells. No intracellular fluorescence was observed throughout the experiment, indicating that the AAZ binding to CAIX was not followed by a significant receptor-mediated endocytosis. In a control experiment, compound 2b, devoid of the targeting moiety, showed no interaction with CAIX-positive SKRC-52 cells (figure III.2).

Figure III.2 Confocal microscopy image of CAIX-expressing SKRC-52 cells after exposure to targeted dye conjugate 2a (120 nM) at different incubation times. The conjugate is mainly bound to the cell surface. Alternatively, SKRC-52 cells were exposed to untargeted dye conjugate 2b (120 nM) for 30 min. CAIX-negative HEK 293 cells were also exposed to targeted dye conjugate 2a (120 nM) for 30 min. No cell surface binding can be detected for both the negative controls. Red = Alexa594 derivatives staining; Blue = Hoechst 33342 staining.

The tumor-targeting properties of acetazolamide derivatives were characterized using both radioactive and near-infrared fluorescence methods in nude mice bearing subcutaneously-grafted SKRC-52 tumors.
Compound 1, labeled with $^{99m}$Tc, was injected intravenously at doses ranging between 70 nmol/Kg and 560 nmol/Kg. A preferential tumor uptake was observed 6 hours after intravenous administration at all doses (figure III.3A).

![Figure III.3](image)

**Figure III.3** (A) Organ distribution of $^{99m}$Tc radiolabeled compound 1 injected at different doses in BALB/c nu/nu mice bearing SKRC-52 xenografts (n = 5 per group). The data, expressed as mean % Injected Dose/gram of tissue ± SD (%ID/g ± SD), correspond to the 6 hours time point after the intravenous administration of the radiolabeled compound; (B) Near-infrared fluorescence imaging evaluation of the targeting performance of the IRDye680RD conjugate 3a in BALB/c nu/nu mice bearing SKRC-52 xenografts.

However, a progressive decrease in percent injected dose per gram (%ID/g) of tumor was detected, reflecting target saturation at the highest dose. Interestingly, compound uptake in normal organs (e.g., liver, lung, kidney, intestine, stomach) also decreased at higher doses. These biodistribution results suggested that doses around 200-300 nmol/Kg may
represent a good compromise between high tumor uptake and low accumulation in normal tissues. The near-infrared fluorescent derivative 3a, administered at 250 nmol/Kg, was shown to preferentially accumulate in the tumor at various time points, between 5 min and 6 hours (figure III.3B). These results are in keeping with the ones previously reported by our group with similar fluorescently-labeled derivatives [88, 143], indicating that the acetazolamide moiety can deliver different types of payloads to CAIX-positive tumors.

Prior to therapy studies, the stability of acetazolamide-drug conjugates 4a and 5a was investigated in vitro at 37 °C, both in phosphate-buffered saline solution (PBS) and in mouse serum (figure III.4).

**Figure III.4** Stability of pro-drugs 4a and 5a in PBS, pH 7.4 (A) and in mouse serum (B) at 37 °C as determined by LC-MS. Dipeptide derivatives of both types of payload were found to be highly stable in saline solution (t_{1/2} > 48 hours). A lower stability was observed in mouse serum, but at least 70% of the compounds were still intact after 6 hours of incubation.

For both compounds, a half-life in PBS >48 hours was observed. A lower stability was seen in mouse serum, but >70% of both conjugates were intact after 6 hours (i.e., a sufficiently long time, compared to the circulatory half-life of acetazolamide derivatives in blood, which is typically shorter than 15 min) [88]. Mass spectrometric analysis of compound 4a revealed free MMAE as the main release product, while in the degradation profile of compound 5a in mouse serum free PNU-
159682 was not identified, probably due to low sensitivity of the mass spectrometer to a such hydrophobic moiety.

An *in vitro* toxicity study, performed with SKRC-52 cells incubated with the conjugates 4a,b-5a,b or with the corresponding unmodified drugs, confirmed that the acetazolamide coupling had led to the formation of pro-drugs. Indeed, all the conjugates resulted to be less toxic compared to the parental cytotoxic moieties (figure III.5).

![Figure III.5](image)

*Figure III.5* Toxicity of (A) MMAE derivatives 4a and 4b, (B) PNU-159682 derivatives 5a and 5b and the corresponding unmodified payloads towards CAIX-expressing SKRC-52 cells. Cells were incubated for 72 hours in the presence of various concentrations of the test compound at 37 °C. Data points are averages of three experiments. Error bars indicate standard deviations. Cytotoxicity values for targeted and non-targeted conjugates are comparable in these in vitro experiments (IC$_{50}$ values: 485 nM for 4a, 204 nM for 4b, 25 nM for 5a, 26 nM for 5b), confirming the absence of efficient internalization. In all cases, prodrugs were found to be less potent in vitro than the parental free cytotoxic compound (IC$_{50}$ values: 1.5 nM for MMAE, 0.16 nM for PNU-159682).

### 2.2 Therapy experiments

Compounds 4a and 5a were used in therapy experiments with nude mice, bearing subcutaneous SKRC-52 tumors. The maximum tolerated doses of the two pro-drugs were determined by dose escalation experiments and found to be 500 nmol/Kg for compound 4a and 50 nmol/Kg for compound 5a (data not shown). The MMAE-conjugate 4a, used at a dose of 250 nmol/Kg with ten daily administrations, exhibited a potent anti-tumor activity effect (p<0.0001 at day 26, compared to the control
groups), while compound 4b (devoid of the acetazolamide moiety and serving as negative control) did not retard tumor growth (figure III.6A).

![Graphs A, B, C, and D showing tumor size and body weight change over time for different treatments.](image)

**Figure III.6** Therapeutic activity of compound 4a (A; 250 nmol/Kg) and 5a (C; 25 nmol/Kg) in BALB/c nu/nu mice bearing SKRC-52 xenografts. In the experiments, drug derivatives devoid of the acetazolamide moiety were used as negative controls (4b and 5b). The graphs in (B) and (D) show the changes in body weight for the treated animals. The statistical analysis of the therapy results, comparing the therapeutic outcome with acetazolamide derivatives and the data obtained with 4b and 5b compounds, indicate superior efficacy of the acetazolamide-based conjugates. **** indicates p<0.0001; *** indicates p<0.001; ** indicates p<0.01; * indicates p<0.05.

In these experimental conditions, no acute toxicity and no significant weight loss was observed for the group of mice treated with the acetazolamide-MMAE conjugate 4a (figure III.6B). One out of five mice enjoyed a durable complete response (with no tumor regrowth for the next four months), while lesions started to regrow in the remaining four mice one week after the last injection.
The anti-tumor efficacy of the PNU-159682 conjugate 5a was investigated at a dose of 25 nmol/Kg (figure III.6C). Also in this case, the conjugate 5a exhibited a potent anti-tumor effect, while the negative control compound 5b did not inhibit tumor growth, confirming the crucial role of the acetazolamide moiety for the potentiation of drug activity. An additional group of animals was injected with equimolar doses of compound 5a and the acetazolamide derivative 3a, leading to a slight decrease in therapeutic activity and tolerability (figure III.6C and II.6D). The treatment of mice with PNU-159682 derivatives was associated with higher toxicity compared to the results obtained with MMAE conjugates, but the 5a treatment group completely recovered from a reversible 10% body weight loss (figure III.6D). At day 40, a second therapeutic cycle of four injections was performed, which did not lead to the disappearance of neoplastic lesions. In order to investigate the reasons for the absence of therapeutic activity in the second treatment cycle, mice were injected with the fluorescently-labeled acetazolamide derivative 3a (250 nmol/Kg) which exhibited only a faint tumor accumulation (figure III.7A), in stark contrast to pre-dosing results (figure III.3B). An immunofluorescence analysis of resected tumors confirmed that cancer cells remained strongly positive for CAIX (figure III.7B), suggesting that a reduction in vascular permeability after therapy may have impaired drug accumulation in the neoplastic lesions.
Figure III.7 Analysis of tumor targeting after therapy experiments. (A) Imaging of a mouse, carrying a subcutaneously-grafted SKRC-52 tumor, with 250 nmol/Kg of the fluorescent acetazolamide derivative 3a, performed 34 days after the beginning of therapy with compound 5a. A striking difference in tumor uptake compared to the data of Figure III.3B can be observed. (B) Microscopic analysis of representative sections of SKRC-52 tumors, before and after therapy with the acetazolamide derivative 5a. Green = CAIX staining; Red = CD31 staining; Yellow = overlap of the two colors; Blue = DAPI staining.

3. Discussion

Acetazolamide derivatives selectively localize to renal cell carcinoma xenografts in mice, which express CAIX as membrane protein. The interaction of these compounds with CAIX-expressing cells has been investigated at the cellular level, by means of fluorescence microscopy experiments. The latter showed that acetazolamide-bearing conjugates bind to CAIX on the cell membrane without triggering a receptor-mediated endocytosis, in agreement with previous findings [49, 87].
A pool of AAZ-based small molecule-drug conjugates has been prepared by our group in recent years: different types of cytotoxic drugs (DM1, MMAE or PNU-159682) were attached to acetazolamide via cleavable linkers (i.e., disulfide bond or valine-citrulline peptide). These SMDCs were found to have similar serum half-lives, which were sufficiently high to exhibit therapeutic benefits in vivo. In particular, the new valine-citrulline-bearing compounds 4a and 5a exhibited a half-life of ~ 7 hours in serum, which is substantially longer than the circulatory half-life of the radiolabeled analogue 1.

While drugs commonly used for the treatment of metastatic kidney cancer (e.g., sorafenib, sunitinib) did not display any detectable activity against SKRC-52 tumors [49], acetazolamide derivatives of MMAE (a tubulin inhibitor) and of PNU-159682 (a nemorubicin metabolite) could suppress tumor growth, sometimes mediating a complete tumor eradication. The removal of the acetazolamide moiety from the linker-payload combinations abrogated therapeutic activity in all experimental systems, confirming that the ligand-based delivery of cytotoxic drugs to the extracellular tumor environment represents a strict requirement for anticancer efficacy. Both compounds 4a and 5a described in this chapter feature a linker containing a valine-citrulline moiety, which was previously believed to be particularly suited for the intracellular release of drug payloads, due to the action of lysosomal cathepsin B [156]. This protein is a primarily intracellular protease that can be secreted extracellularly by dying cells, but also by living tumor cells to initiate extracellular proteolytic cascades and to enable tumor cell proliferation [100]. Our therapy data suggest that the presence of cathepsin B in the extracellular tumor environment is sufficient for the efficient liberation of cytotoxic drug moieties, which can then diffuse into surrounding tumor cells.

Nude mice do not represent a perfect model for the assessment of anticancer agents for a number of reasons [157], including their lack of functional T cells. It has recently been shown that certain cytotoxic agents
can mediate an immunogenic tumor cell death [158, 159]. In addition, certain immunostimulatory agents (e.g., antibody-cytokine fusion proteins, immunological check-point inhibitors) may potently synergize with some cytotoxic agents [160-162].

In spite of the fact that a strong suppression of tumor growth was observed both for compounds 4a and 5a, cancer cures were rare. Interestingly, treatment of mice with a second cycle of 5a therapy did not cause a second tumor regression (figure III.6). Macroscopic and microscopic imaging data suggest that the tumor uptake of acetazolamide derivatives is reduced after therapy, probably as a result of changes in vascular permeability at the neoplastic site, while cancer cells remain CAIX-positive (figure III.7). It will be interesting to investigate whether similar findings can be observed with other classes of targeted cytotoxics (e.g., with ADC products).

The potent therapeutically activity observed with compounds 4a and 5a suggests that these products may provide a benefit to patients with kidney cancer, as the majority of clear cell renal cell carcinomas are strongly positive for this target [66, 70, 152]. The biodistribution profiles of acetazolamide derivatives compares favorably with the ones previously reported for other ligands [146-149]. In particular, we were pleased to see that %ID/g values in the tumor were higher than the corresponding values in the kidney (the main organ for drug clearance), even as early as one hour after intravenous administration of the product [88]. A nuclear medicine trial in patients with metastatic renal cell carcinoma is currently planned for ⁹⁹mTc-labeled acetazolamide 1 (EUDRACt n° 2016-004909-13). That study will provide essential information, regarding the tumor:organ ratios that can be expected in cancer patients and in mouse models of the disease.
IV. LINKER STABILITY INFLUENCES THE 
ANTI-TUMOR ACTIVITY OF 
ACETAZOLAMIDE-DRUG CONJUGATES FOR 
THE THERAPY OF RENAL CELL 
CARCINOMA


1. Introduction

The majority of low molecular weight drugs, commonly used for cancer chemotherapy, do not preferentially localize to solid tumors in mice [134, 135] and patients [22]. This unfavorable pharmacokinetic behavior may lead to undesired toxicity to normal organs and may limit the dose escalation to therapeutically active regimens. In order to improve the therapeutic activity of anti-cancer chemotherapy, the coupling of cytotoxic agents to monoclonal antibodies has been proposed and experimentally explored, at the preclinical and clinical level [27, 141, 164, 165]. Several monoclonal antibody-drug conjugates (ADCs) have exhibited promising therapeutic results in mouse models of cancer and two products (Adcetris™ and Kadcyla™) have gained marketing authorization for the treatment of certain malignancies in patients. However, the clinical performance of ADC products is often limited by the slow extravasation of the antibody molecule [166], suboptimal in vivo selectivity [167] and premature drug release [168-170], which may contribute to undesired toxicities and limit therapeutic efficacy.
Small molecule-drug conjugates (SMDCs), capable of selective localization at the tumor site, have been proposed as an alternative to ADCs [171]. Small organic ligands, specific to accessible tumor-associated antigens, extravasate and diffuse into tissues more rapidly than antibodies [44, 172]. These pharmacokinetic characteristics may lead to favorable tumor:blood and tumor:organ ratios at early time points (e.g., few hours) after intravenous administration [52]. Small molecule ligands have been used for the selective in vivo targeting of tumors expressing the folate receptor [173, 174], prostate-specific membrane antigen [48, 52] and somatostatin receptors [175]. A number of SMDC products are currently being investigated in clinical trials [8].

Carbonic anhydrase IX (CAIX) is a membrane-bound homodimeric enzyme, which is undetectable in most normal adult tissues [73]. CAIX can be found in certain gastro-intestinal structures (e.g., stomach, duodenum and gallbladder) [176], albeit in a catalytically inactive form [177], and in hypoxic tissues [72]. Interestingly, CAIX is also strongly expressed in the majority of kidney cancers, as a result of von Hippel-Lindau mutations and the in vivo ligand-based targeting of this enzyme is more efficient in tumors, compared to normal organs [83]. Moreover, the antigen has been reported to be abundant in a subset of patients with different cancers (i.e., lung, colorectum, stomach, pancreas, breast, cervix, bladder, ovaries, brain, head and neck and oral cavity [178]) with an over-expression at the growing front of the tumor [179].

Even though CAIX has previously been claimed to be an internalizing antigen and has been considered for industrial ADC product development activities [84], our lab has experimentally shown that the protein remains membrane-bound and does not efficiently internalize upon small-ligand binding [49, 132].

Acetazolamide is a small heteroaromatic sulfonamide, which binds to various carbonic anhydrases with high affinity. Derivatives of acetazolamide containing multiple charges do not efficiently cross the cell
membrane and are restricted for binding to membrane-accessible carbonic anhydrases (i.e., CAIX, but also potentially CAIV and CAXII). We have previously shown that certain acetazolamide derivatives selectively localize to renal cell carcinomas in vivo [49, 87, 88] and that those ligands can be used for the selective delivery of highly cytotoxic maytansinoids (e.g., DM1) to kidney tumors. Interestingly, the use of disulfide linkers for the coupling of DM1 to acetazolamide allows an efficient and selective drug release at the tumor site, where dying cells release large amounts of glutathione and other reducing agents. Indeed, disulfide linkers have been proposed as selective modules for drugs release also with antibody-drug conjugates [33, 150] and with polymer-drug conjugates [180].

In this chapter, we describe the synthesis and characterization of four SMDCs, in which the acetazolamide moiety was coupled to monomethyl auristatin E (MMAE, the payload in Adcetris™) via cleavable linkers, featuring four different dipeptide structures. We observed that valine-citrulline and valine-alanine linkers were more stable in serum, compared to the charged valine-lysine and valine-arginine structures. Interestingly, the two most stable SMDCs were also the most therapeutically active products, when tested in mice with xenografted SKRC-52 tumors. These findings are of potential therapeutic significance, as the CAIX targeting agents could be considered for applications in humans. Furthermore, our data indicate that potent therapeutic activity can be achieved in vivo, using non-internalizing ligands and linkers, which had previously been postulated to be processed only in intracellular compartments, by cathepsin B cleavage [181, 182].
2. Results

2.1 Synthesis and in vitro characterization of acetazolamide-based drug conjugates

MMAE-dipeptide substrates, bearing a self-immolative linker and a Michael-acceptor maleimido moiety (suitable for conjugation with thiols), were synthesized in solution, as described in chapter VII - Appendix II (figure IV.1). A derivative of acetazolamide (a heteroaromatic sulfonamide, capable of CAIX binding), bearing an Asp-Arg-Asp-Cys tetrapeptide moiety (compound 1 in figure IV.1), was then coupled to the MMAE-dipeptide-maleimido derivative, yielding products 2-5. These compounds featured valine-alanine, valine-lysine, valine-arginine or valine-citrulline dipeptide structures as cleavable moieties, respectively, which can subsequently trigger the release of the MMAE cytotoxic moiety (figure IV.1). Compound 1 was prepared by solid phase synthesis, installing the acetazolamide moiety onto the Asp-Arg-Asp-Cys tetrapeptide linker by a copper-catalyzed azide-alkyne cycloaddition “on resin” (figure IV.1).

![Figure IV.1 Synthesis of Acetazolamide-based SMDCs (compounds 2-5). REAGENTS AND CONDITIONS: a) SPPS performed according to previously reported procedures [49]; b) 1, maleimidocaproyl-Val[Cit/Ala/Arg/Lys]-MMAE, PBS/DMF 2:1, 3 hours, RT, 50-90%.

The stability of the acetazolamide-MMAE conjugates 2-5, bearing different cleavable dipeptide sequences, was analyzed by LC/MS. The
methodology detected the amounts of intact compounds at various time points, upon incubation at 37 °C in either phosphate-buffered saline (PBS) or mouse serum (figure IV.2).

![Figure IV.2 Comparison of the stability of 2-5 in PBS (A) and in mouse serum (B) at 37 °C as determined by LC-MS. All SMDCs were found to be highly stable in saline solution (t_{1/2} > 96 hours), whereas they showed lower half-lives when incubated in mouse serum. In particular, conjugates AAZ-ValArg-MMAE 4, AAZ-ValLys-MMAE 3, AAZ-ValCit-MMAE 5 and AAZ-ValAla-MMAE 2 showed increasing stability in the order (i.e., t_{1/2} = 1.8, 8.2, 11.2 and 23 hours, respectively), highlighting the effect of different linkers on biological properties.

While no degradation of the tested conjugates was observed in PBS (t_{1/2} > 96 hours in at 37 °C), compounds 2-5 exhibited various degrees of chemical stability in mouse serum at 37 °C. Since free MMAE was detected as end product in LC/MS analysis, in parallel to the progressive disappearance of the conjugates, the stability profiles of figure IV.2 can be related to the differential susceptibility to proteolytic cleavage of the corresponding dipeptide linkers. In particular, conjugates 2 and 5 (i.e., featuring the valine-alanine and valine-citrulline linkers, respectively) showed the highest stability (t_{1/2} = 23 hours and 11.2 hours), compared to the more labile compounds 3 (valine-lysine; t_{1/2} = 8.2 hours) and 4 (valine-arginine; t_{1/2} = 1.8 hours).

These data are in agreement with previous publications, which reported the lower stability of linkers featuring protonable side chains (e.g., arginine, lysine) in serum or in the presence of proteases. Considering
the rapid clearance of acetazolamide derivatives from the blood (with half-lives which are typically shorter than 15 minutes), the serum stability of compounds 2-5 is sufficient for in vivo pharmacodelivery applications, favoring a preferential tumor uptake and a slow drug release at the site of disease.

The antiproliferative activity of conjugates 2-5 was tested in vitro against CAIX-positive renal carcinoma SKRC-52 cells, using the free MMAE payload as reference compound (figure IV.3). Cells were incubated for 72 hours in the presence of different concentrations of the tested compounds.

![Graph](image)

**Figure IV.3** Toxicity of MMAE derivatives 2-5 compared to the unmodified payload towards CAIX-expressing SKRC-52 cells. Cells were incubated for 72 hours in the presence of crescent concentrations of the test compound at 37 °C. The experiments were performed in triplicate and data points are averages values. Error bars indicate standard error of the means (SEM). Cytotoxicity values (IC$_{50}$) for AAZ-ValAla-MMAE (2; 69 nM) and AAZ-ValCit-MMAE (5; 171 nM) are comparable in these in vitro experiments. Both compounds demonstrate low toxicities when compared to the free payload MMAE (0.9 nM) confirming their pro-drug behavior and the absence of efficient internalization. Conjugates AAZ-ValLys-MMAE (3; 1.6 nM) and AAZ-ValArg-MMAE (4; 2.1 nM) show cytotoxicity comparable to MMAE (0.9 nM), indicating possible parental free cytotoxic release during the incubation time.

Under this experimental procedure, the free MMAE drug was found to inhibit cell proliferation at sub-nanomolar concentrations (IC$_{50}$ = 0.9 nM).
Only a small decrease in cytotoxic activity was observed for the pro-drugs AAZ-ValLys-MMAE (3) and AAZ-ValArg-MMAE (4), which showed IC\textsubscript{50} values of 1.6 nM and IC\textsubscript{50} = 2.1 nM, respectively. By contrast, compounds AAZ-ValAla-MMAE (2) and AAZ-ValCit-MMAE (5) were found to be substantially less potent than the free drug \textit{in vitro} (i.e., IC\textsubscript{50} values of 69 nM and 171 nM, respectively). This observation is in keeping with the enhanced serum stability of the valine-alanine and valine-citrulline linkers, compared to valine-lysine and valine-arginine (figure IV.2B).

2.2 Therapy experiments

The therapeutic activity of the acetazolamide derivatives 2-5 was tested in immunodeficient BALB/c nude mice, xenografted subcutaneously with SKRC-52 [49]. Multiple intravenous injections at equivalent molar doses (250 nmol/Kg) were performed following the schedule indicated in figure IV.4.

![Figure IV.4](attachment:image.png)

\textbf{Figure IV.4} Therapy experiment with AAZ-dipeptide-MMAE derivatives 2-5 (250 nmol/Kg) in BALB/c nude mice bearing SKRC-52 xenografts. Graph (A) compares the therapeutic activity of the SMDCs with different dipeptide linker. In graph (B) percentage changes of body weight during the experiment are represented. The statistical analysis of the therapy result reveals superior efficacy of the acetazolamide derivatives bearing the ValAla-MMAE (2) and the ValCit-MMAE (5), when compared with AAZ-ValLys-MMAE (3) and AAZ-ValArg-MMAE (4) groups. **** indicates p<0.0001; *** indicates p<0.001; ** indicates p<0.01; * indicates p<0.05.
All conjugates 2-5 significantly inhibited tumor growth, compared to saline treatment (p<0.0001) (figure IV.4A). The acetazolamide derivatives, featuring valine-alanine (2) and valine-citrulline (5) as cleavable linkers, displayed the best anti-tumor efficacy (p<0.0001). None of the study groups exhibited a body weight loss of more than 10%, which was comparable to the one observed in the saline treatment group (figure IV.4B). A second cycle of four injections at the same dose was administered to mice, which had enjoyed the largest tumor growth inhibition as a result of AAZ-ValAla-MMAE and AAZ-ValCit-MMAE treatment. However, the second cycle of injections did not result in a tumor regression (figure IV.4A). In order to better characterize the mechanism of resistance to drug action at late time points, mice were treated with a near-infrared fluorescent derivative of acetazolamide (compound 6) (figure IV.5A). For all treatment groups, the imaging data confirmed the ability of acetazolamide to selectively target tumor lesions at the end of the therapy experiment (figure IV.5B). After sacrifice of the mice, tumor sections were stained with anti-CAIX antibodies, confirming that tumor cells had not lost antigen expression (figure IV.5C).
Figure IV.5 (A) Chemical structure of IRdye680RD fluorophore labeled with acetazolamide for the targeting of CAIX (compound 6). (B) Microscopic analysis of representative sections of SKRC-52 tumors, after treatment with acetazolamide-based SMDCs 2-5 or with vehicle. Green = CAIX staining; Red = CD31 staining for blood vessels; Blue = DAPI staining for nuclear structures. Tumors samples from all the groups resulted to be positive for Carbonic Anhydrase IX. (C) Imaging of representative SKRC-52 bearing mice with 250 nmol/Kg of the fluorescent acetazolamide derivative 6, performed after therapy study with vehicle or with compounds 2-5. Imaging was performed after therapy study, when the animals reached termination criteria (Vehicle group: day 21; groups 3 and 4: day 28; groups 2 and 5: day 40; see also Figure 4). Selective tumor uptake of compound 6 can still be observed in all the treatment groups, as compared to the vehicle group.
3. Discussion

Patients with metastatic renal cell carcinoma (RCC) typically receive sunitinib or sorafenib as first-line treatment, but durable objective responses are rarely observed [183]. Both of these drugs fail to preferentially localize on tumor lesions [138, 184] and are poorly active in mouse models of RCC. Interestingly, sunitinib and sorafenib treatment in mice bearing SKRC-52 tumors was not significantly better than saline treatment [49], while the acetazolamide derivatives 2 and 5 exhibit a substantial and prolonged tumor regression (figure IV.4).

In spite of their therapeutic activity and good tolerability profile, the conjugates AAZ-ValAla-MMAE (2) and AAZ-ValCit-MMAE (5) were not able to cure mice with SKRC-52 tumors, when used at the maximum tolerated dose of 250 nmol/Kg. This dose is compatible with an efficient ligand localization at the tumor site, as revealed by quantitative biodistribution studies with radio-labeled acetazolamide derivatives [88, 132]. The lack of sufficient therapeutic activity at later time points was not associated with loss of ligand-based targeting or of CAIX expression by tumor cells (figure IV.5). The use of human tumors grafted in nude mice does not allow to study the contribution of the adaptive arm of the immune system to tumor rejection. In the future, it will be interesting to study the therapeutic activity of compounds 2 and 5 in syngeneic tumor models, expressing CAIX. Potentially, these agents could synergize with other interventional modalities, such as external beam radiation [185] or certain immunostimulatory regimens [186] in promoting tumor rejection.

Developed in the late 90s [187], the valine-citrulline linker is one of the most broadly used dipeptide linkers in the ADC field, being included in the majority of MMAE-based ADCs in clinical studies [102], including brentuximab vedotin [101]. Interestingly, the valine-alanine dipeptide has been used in two pyrrolobenzodiazepine (PBD)-based ADCs that are currently being evaluated in the clinic. Notably, the CD33-targeting ADC vadastuximab talirine (SGN-CD33A) has recently entered pivotal phase 3
CASCADE study in patients with newly diagnosed acute myeloid leukemia (AML). For all these ADC products, the proposed mechanism of action featured selective antibody internalization into antigen-positive cells, followed by intracellular release of the payload. We have shown, however, that both ADC linkers based on disulfides or on peptide structures can efficiently liberate drug in the tumor extracellular environment, with a therapeutic benefit in rodent models of cancer [33, 150].

Some examples of SMDC products, featuring protease-cleavable linkers (e.g., incorporating the valine-citrulline [188, 189], and valine-alanine [190, 191] dipeptides) have previously been reported. Together with the uptake in healthy organs, an insufficient in vivo stability of the linker may contribute to off-target toxicities and loss of efficacy [168-170]. Our comparative evaluation of acetazolamide-peptide-conjugates indicates a correlation between linker stability and activity. Acetazolamide derivatives can achieve tumor:blood ratios >100:1 six hours after intravenous administration [88, 132]. SMDC products with a serum stability of at least few hours (t_{1/2} >10 hours for valine-citrulline and valine-alanine derivatives) are thus able to preferentially localize at the cancer site, before releasing their payload in the tumor environment. The growing body of experimental evidence, documenting a preferential drug release in the extracellular tumor space using non-internalizing conjugates equipped with disulfide or peptide linkers [33, 49, 132, 150], indicates that sufficient amounts of reducing agents (e.g., glutathione) and of proteases (e.g., cathepsin B) are set free as a result of malignant cell death.

In order to facilitate a clinical translation of the best-performing MMAE conjugates, it will be essential to characterize the tumor-targeting ability of acetazolamide derivatives in patients with metastatic kidney cancer. We have recently described an acetazolamide derivative, which can be labeled with $^{99m}$Tc and which has recently completed GMP manufacturing [88, 132]. In full analogy to monoclonal antibodies [142, 192], Nuclear
Medicine studies in patients have the potential to accompany product development activities for SMDCs.
V. ENHANCED THERAPEUTIC ACTIVITY OF NON-INTERNALIZING SMDCs TARGETING CAIX IN COMBINATION WITH TARGETED IL-2

This chapter corresponds to the paper “Enhanced therapeutic activity of non-internalizing Small Molecule-Drug Conjugates targeting carbonic anhydrase IX in combination with targeted interleukin-2.” by S. Cazzamalli, B. Ziffels, F. Widmayer, P. Murer, G. Pellegrini, F. Pretto, S. Wulhfard & Dario Neri. 2018 Accepted for publication on Clinical Cancer Research.

1. Introduction

Cytotoxic drugs are frequently used for cancer chemotherapy [12], but the unfavorable tissue distribution properties of these molecules often prevent dose escalation to therapeutically active regimens [9]. A suboptimal uptake of anti-cancer drugs in the neoplastic masses has been confirmed in cancer patients, using isotope-labeled preparations of drugs and positron emission tomography methodologies [133]. This pharmacokinetic limitation of conventional chemotherapy makes it more difficult to induce objective responses in patients and contributes to side effects [193].

In order to overcome the limitations of conventional cytotoxic agents, it is attractive to couple potent drugs to monoclonal antibodies [10, 27] or to small organic ligands [8, 9], which recognize a cognate antigen in the tumor environment and may therefore facilitate a preferential uptake at the site of disease. While it has long been thought that internalization of drug conjugates into the tumor cell may represent an indispensible requirement for potent anti-cancer activity [25], experimental evidence suggests that it may be possible to release cytotoxic payloads in the tumor extracellular space [33, 49, 96, 103, 151]. Three antibody-drug
conjugates (ADCs) have gained marketing authorization (Kadcyla™, Adcetris™ and Besponsa™) for cancer therapy [194-196], while small molecule-drug conjugates (SMDCs) are still being investigated both at the preclinical and clinical level [8, 9]. SMDCs could offer certain advantages compared to ADCs, such as a more rapid and uniform diffusion into the tumor mass [145], a lower cost-of-goods [197] and lack of immunogenicity [198].

Certain folate analogues [146], prostate-specific membrane antigen binders [148], somatostatin antagonists [149] and carbonic anhydrase IX ligands [49, 63, 88] have been considered as delivery vehicles for SMDC development activities, based on promising tumor-targeting performance in Nuclear Medicine studies [50, 53, 199] and on encouraging preclinical results.

Carbonic anhydrase IX (CAIX) is a homodimeric membrane protein that is virtually undetectable in most normal adult tissues, exception made for certain gastrointestinal structures [152]. However, CAIX is up-regulated in hypoxic cells and is over-expressed in approximately 90% of clear cell renal cell carcinomas, as a result of von Hippel-Lindau inactivation [70]. Interestingly, CAIX is also strongly expressed in a portion of patients with other malignancies, including colorectal, urothelial, lung, stomach, pancreas, breast, head and neck, bladder, ovaries, brain and cervix cancer [178]. Antibodies specific to CAIX efficiently localize to metastatic renal cell carcinoma lesions but not to normal gastrointestinal structures, as revealed by immuno-PET studies in cancer patients [200]. Acetazolamide (an approved drug for the treatment of idiopathic intracranial hypertension, metabolic alkalosis and glaucoma) exhibits a high binding affinity to CAIX [201]. The development of charged derivatives of acetazolamide prevents the crossing of the cellular membrane and the binding to abundant intracellular carbonic anhydrase isoforms [49]. Quantitative biodistribution studies performed in nude mice, bearing SKRC-52 human kidney cancer xenografts, revealed that $^{99m}$Tc-
labeled charged derivatives of acetazolamide would achieve tumor:blood ratios of ~100:1, six hours after intravenous administration [88, 132]. Our group has previously described the synthesis and potent anti-tumor activity of non-internalizing anti-CAIX SMDCs, based on acetazolamide conjugates to DM1, monomethyl auristatin-E or the ultrapotent nemorubicin derivative PNU-159682, featuring the use of cleavable disulfide bridges or dipeptide linkers [49, 132, 163]. However, we have recently observed that the discovery of an acetazolamide derivative with a higher affinity for CAIX (hereafter named AAZ⁺) [63] would lead to an improved tumor targeting performance, compared to acetazolamide derivatives [64, 88]. Encouraged by favorable biodistribution results, we here describe the synthesis and anti-cancer activity of a novel SMDC, based on the high-affinity AAZ⁺ CAIX-ligand, a cleavable peptide linker and monomethyl auristatin E (MMAE) as cytotoxic payload. We observed that the new SMDC was well tolerated and potently active. Durable complete cancer eradications could be observed in all treated mice when the new SMDC product was combined with L19-IL2, a clinical-stage fusion protein that delivers interleukin-2 to the alternatively-spliced EDB domain of fibronectin, typically expressed in the sub-endothelial extracellular matrix of tumor blood vessels [202-206]. Collectively, our results provide a rationale for the clinical development of acetazolamide-drug conjugates for the treatment of patients with CAIX-positive malignancies, as well as for their combination with immunostimulatory drugs, such as L19-IL2.
2. Results

2.1 Synthesis and in vitro activity of acetazolamide SMDC derivatives

The tumor-targeting performance of AAZ\textsuperscript{+}, an affinity-matured version of acetazolamide discovered using encoded self-assembling chemical libraries and previously characterized by near-infrared fluorescence imaging [63], was confirmed in mice with subcutaneously-grafted SKRC-52 tumors, using a radiolabeled AAZ\textsuperscript{+} derivative. The product accumulated with 40% ID/g in the tumor mass six hours after intravenous administration (with a tumor:blood ratio of 80:1), while a similar compound featuring acetazolamide (AAZ) as CAIX binder exhibited a 18% ID/g at the same time point (figure V.1). Encouraged by these favorable biodistribution results, we synthesized a novel SMDC product, featuring the AAZ\textsuperscript{+} structure as tumor-homing moiety.
Figure V.1 Chemical structures and organ distribution of $^{99m}$Tc-radiolabeled AAZ and AAZ$^+$ (compounds 7 and 8) in BALB/c nu/nu mice bearing SKRC-52 xenografts (n = 3 per group). The data, expressed as mean % Injected Dose/gram of tissue ± SD (%ID/gram), correspond to the 6 hours time point after the intravenous administration of the radiolabeled compound.
SMDC products were designed following the general ligand-spacer-linker-drug scheme depicted in figure V.2.

![Diagram of SMDC products](image)

1 - (AAZ-ValCit-MMAE)  
2 - (AAZ⁺-ValCit-MMAE)  
3 - (Negative control)

**Figure V.2** General scheme of Small Molecule-Drug Conjugates (SMDCs) and chemical structures of CAIX-targeting SMDCs. Compounds display an acetazolamide targeting ligand (AAZ; compound 1), an affinity matured version of acetazolamide (AAZ⁺; compound 2) or an amide (serving as negative control; compound 3). All products feature the cytotoxic payload MMAE, an Asp-Arg-Asp-Cys peptide spacer, a Valine-Citrulline (ValCit) dipeptide cleavable linker and a p-amino benzyl (PAB) self-immolative linker.

The CAIX-ligand acetazolamide was installed by copper-catalyzed azide-alkyne cycloaddition (“Click” reaction) on solid phase onto different peptides, which featured the presence or the absence of a 4,4-bis(4-hydroxyphenyl)valeric acid moiety, responsible for high-affinity CAIX binding [63]. The resulting SMDCs 1 and 2 were subsequently cleaved and purified. In addition, compound 3 (which served as negative control) was synthesized in a similar way, replacing the acetazolamide moiety with an amide and thus generating a structure devoid of CAIX binding (figure V.2). Reduction of a C-terminal cysteine residue allowed the
coupling to a linker-payload, featuring a valine-citrulline cleavable linker, a self-immolating para-aminobenzyl carbamate and the potent anti-tubulin monomethyl auristatin E (MMAE), which is also used in Adcetris™. All products were obtained as highly pure (>95% purity by UPLC) lyophilized material, characterized and compared for the binding to the cognate antigen (figure V.3), and used for the subsequent biological assays.

**Figure V.3** SPR sensorgrams and fitting data of a dilution series of (A) AAZ-ValCit-MMAE (1; 1 µM to 250 nM in steps of 1:2) and (B) AAZ⁺-ValCit-MMAE (2; 500 nM to 125 nM in steps of 1:2) binding to immobilized human carbonic anhydrase IX (hCAIX). Binding kinetics were analyzed with the Biacore™ S200 evaluation software version using a 1:1 Langmuir binding model.

The cytotoxic activity of compound AAZ⁺-ValCit-MMAE 2 was tested on SKRC-52 cells and compared to the anti-proliferative properties of free MMAE, used as reference compound (figure V.4).
Figure V.4 Cells were incubated for 72 hours in the presence of various concentrations of the test compound at 37°C. Data points are averages of three experiments. Error bars indicate standard deviations. Compound 2 behaves as a prodrug, being found to be less potent in vitro than the corresponding free drug (IC$_{50}$ value: 440 nM for 2, 0.9 nM for MMAE). This experimental data confirms the absence of internalization of the affinity matured AAZ$^+$ ligand for CAIX.

As expected, Compound 2 acted as a pro-drug and exhibited a substantially higher IC$_{50}$ value (440 nM) compared to MMAE (IC$_{50}$ = 0.9 nM), in line with what had previously been published several non-internalizing SMDC derivatives bearing the same linker-payload structure.

2.2 Therapy experiments in SKRC-52 renal cell carcinoma model

We compared the anti-cancer activity of compounds AAZ-ValCit-MMAE (1) and AAZ$^+$-ValCit-MMAE (2) in BALB/c nude mice, bearing subcutaneously-grafted SKRC-52 renal cell carcinomas. Compound 3, devoid of the CAIX-targeting moiety but otherwise identical to compound 2, served as negative control. The SMDCs were administered at the maximal tolerated dose, which had been found to be 250 nmol/Kg in preliminary experiments (data not shown), following the schedule depicted in figure V.5. A fourth group of mice (“presaturation” group) was injected with a 50-fold higher dose (12.5 µmol/Kg) of ligand AAZ$^+$, which was directly followed by the administration of AAZ$^+$-ValCit-MMAE (2). Both compounds 1 and 2 showed a potent anti-cancer activity (p<0.0001
compared to the group of mice treated with vehicle), while negative control compound 3 did not display a difference from the vehicle-treated group. Compound 2 was very well tolerated (figure V.5B) and exhibited the most potent tumor growth retardation effect, but none of the mice enjoyed a durable complete response.

![Graphs A and B](image)

**Figure V.5** Comparison of the *in vivo* efficacy of AAZ-ValCit-MMAE and AAZ⁺-ValCit-MMAE (compounds 1 and 2) in BALB/c nu/nu mice bearing subcutaneous SKRC-52 renal cell carcinomas. Cytotoxic derivative 3 devoid of the acetazolamide moiety was used as negative control. All the compounds were injected intravenously at the dose of 250 nmol/Kg per administration. The “presaturation” group was treated with a 50-fold dose of AAZ⁺ ligand (1.25 µmol/Kg; compound 4) directly followed by an administration of compound 2 (250 nmol/Kg). Graph (A) compares the therapeutic activity of the different treatment. Data points represent mean tumor volume ± SEM, n = 4 per group. SMDC 2 based on the affinity matured AAZ⁺ ligand exhibited a superior anti-tumor activity when compared with SMDC 1, based on the non-matured AAZ targeting moiety. The therapeutic efficacy of SMDC 2 was reduced significantly by the presaturation with an excess of free AAZ⁺. In graph (B) percentage changes of body weight during the experiment are represented. **** indicates p<0.0001; ** indicates p<0.01; * indicates p<0.05; ns indicates p>0.05 (2-way ANOVA test, followed by Bonferroni post-test).

In a second therapy experiment performed in the same cancer model, compound 2 (AAZ⁺-ValCit-MMAE) was tested in combination with the clinical-stage antibody-cytokine fusion protein L19-IL2 [203-206]. A selective uptake of L19-IL2 at the tumor site had been confirmed in the
SKRC-52 model, using biodistribution studies and a radioiodinated protein preparation (figure V.6).

![Organ distribution of radioiodinated L19-IL2 in BALB/c nu/nu mice bearing SKRC-52 xenografts (n = 3). The data, expressed as mean % Injected Dose/gram of tissue ± SD (%ID/gram), correspond to the 24 hours time point after the intravenous administration of the radiolabeled protein.](image)

**Figure V.6** Organ distribution of radioiodinated L19-IL2 in BALB/c nu/nu mice bearing SKRC-52 xenografts (n = 3). The data, expressed as mean % Injected Dose/gram of tissue ± SD (%ID/gram), correspond to the 24 hours time point after the intravenous administration of the radiolabeled protein.

While neither L19-IL2 nor compound 2 were able to eradicate SKRC-52 xenografts when used as single agent, the combination of the two agents was well tolerated and induced complete remissions in all mice (figures IV.7 and IV.8). Cured mice were re-challenged after the therapy by a second subcutaneous injection of cancer cells and tumors regrew in all cases, as expected considering the lack of functional T cells in nude mice. Macroscopically, the combination of SMDC with L19-IL2 induced flattening of the tumor site and hematoma formation at the site previously occupied by the solid tumor.
Figure V.7 Therapeutic activity of AAZ\textsuperscript{5}-ValCit-MMAE in combination with the immunocytokine L19-IL2 in BALB/c nu/nu mice bearing SKRC-52 renal cell carcinoma. Nude mice bearing established subcutaneous tumors were treated with AAZ\textsuperscript{5}-ValCit-MMAE (compound 2; 250 nmol/kg; IV; black arrow) and L19-IL2 (2.5 mg/Kg; IV; white arrow), as monotherapy or in a combination regimen. Graph (A) compares the therapeutic activity of the different treatment. Data points represent mean tumor volume ± SEM, n = 5 per group. CR = Complete Responses. In graph (B) percentage changes of body weight during the experiment are represented. **** indicates p<0.0001; *** indicates p<0.001; ** indicates p<0.01 (2-way ANOVA test, followed by Bonferroni post-test).
Day 17 after tumor implantation

Vehicle

L19IL2 (2.5 mg/Kg)

AAZ⁺-ValCit-MMAE (250 nmol/Kg)

AAZ⁺-ValCit-MMAE (250 nmol/Kg) + L19IL2 (2.5 mg/Kg)

**Figure V.8** Photos of BALB/c nude mice bearing subcutaneous SKRC-52 tumors, on day 17 after tumor implantation, after having received different treatments. Potent antitumor activity was observed for L19-IL2 and AAZ⁺-ValCit-MMAE (2), but only the combination of the two agents led to the complete regression of the tumor in the model.
Histologically, xenografts from mice that had received the vehicle or the monotherapy were composed of coalescing sheets and nests of densely packed neoplastic epithelial cells, separated by inconspicuous fibrous stroma and mild infiltration of inflammatory cells (figure V.9).

**Figure V.9** Ex vivo histological and immunofluorescence analysis on SKRC-52 tumor sections following treatment with vehicle, AAZ\(^+\)-ValCit-MMAE (SMDC), L19-IL2 (immunocytokine) or the AAZ\(^+\)-ValCit-MMAE/L19-IL2 combination. Representative images of mice treated with saline, single agents or combination. H&E staining and immunofluorescence analysis of tumor samples are depicted at a 30X and 10X magnification, respectively (scale bars: 100 µm). Green = NKp46 staining; Red = Caspase 3 staining; Blue = DAPI staining. A marked increase in infiltrating inflammatory cells (white arrowheads) is observed following treatment with the AAZ\(^+\)-ValCit-MMAE/L19-IL2 combination. Increased numbers of infiltrating NK cells, associated with higher numbers of neoplastic apoptotic cells, are detected in the same group. All H&E pictures are taken from the center of the xenografts. The homogenous eosinophilic appearance observed in the nuclei of the neoplastic cells from the vehicle xenograft represents a freezing artifact.
Tumors from mice exposed to the combination therapy exhibited instead a marked increase in the number of inflammatory cells (mainly p46-positive NK cells) infiltrating the xenograft. Moreover, combination treatment led to a potent and homogenous induction of apoptosis within the tumor mass, as revealed by cleaved caspase 3 staining (figures IV.9, IV.10 and IV.11).

*Figure V.10* Nude mice bearing subcutaneous SKRC-52 xenografts were treated with the indicated therapeutics. The figure shows representative fluorescent images of tumor sections obtained after 1, 2 or 3 cycles of therapy (as indicated in the scheme above) and stained for NK cells (green, NKp46). Scale bars: 100 µm. Treatment with the SMDC/L19-IL2 combination, but not with single agents injected in a monotherapy setting, leads to the recruitment of spots of NK cells at the tumor site, which is evident in mice treated with three cycles of the combination.
Nude mice bearing subcutaneous SKRC-52 xenografts were treated with the indicated therapeutics. The figure shows representative H&E stainings of tumor sections obtained after 1, 2 or 3 cycles of therapy (as indicated in the scheme above). Increased numbers of inflammatory cells in the xenografts exposed to combination therapy (letter) are already observed following the second cycle.

Infiltration by myeloperoxidase-positive neutrophils and CD68-positive macrophages was low in all groups and restricted to the periphery of the xenografts (figure V.12).
Figure V.12 *Ex vivo* immunohistochemical analysis on SKRC-52 tumor sections following treatment with vehicle, AAZ\(^{+}\)-ValCit-MMAE (SMDC), L19-IL2 (immunocytokine) or the AAZ\(^{-}\)-ValCit-MMAE/L19-IL2 combination. Representative images of mice treated with saline, single agents or combination. Immunohistochemical analysis of tumor samples are depicted at a 20X (scale bars: 50 µm). Red = myeloperoxidase-positive neutrophils; Brown = CD68-positive macrophages. Moderate numbers of neutrophils and macrophages infiltrate the periphery of all xenografts, without meaningful difference between the different therapy groups.

2.3 **Therapy experiments in immunocompetent mice, bearing syngeneic CT26.3E10 tumors**

Approximately 30% of patients with colorectal cancer express high levels of CAIX in their tumor, but the immunocompetent mouse models of the disease tested by our group were negative for the antigen (data not shown). In order to establish an immunocompetent murine model of colorectal cancer expressing CAIX, we stably-transfected colorectal CT26.wt cancer cells with a gene coding for human CAIX and a second gene conferring antibiotic resistance against Neomycin (figure V.13A).
Figure V.13 In vitro and in vivo evaluation of the new murine colorectal carcinoma model CT26.3E10 transfected with the human antigen CAIX. (A) Cloning scheme of human CAIX in pcDNA3.1(+). (B) FACS analysis for expression of human CAIX on SKRC-52, CT26.wt (wild type) and transfected CT26.3E10 cells. Staining was performed with a human anti-CAIX specific antibody and the corresponding signal was amplified with an anti-human AlexaFluor488 secondary antibody. (C) Antigen expression by CT26.3E10 transfected monoclonal cell line checked by immunofluorescence. Green = human CAIX staining by a human anti-CAIX specific antibody; Red = cytoskeleton staining by phalloidin; Blue = nuclei staining (DAPI); Scale bars: 50 μm. (D) Confocal
microscopy analysis on living SKRC-52 CT26.wt and CT26.3E10 cells after exposure to acetazolamide labeled with AlexaFluor594 (AAZ-AlexaFluor594; 5) (120 nM) at 1 hour incubation time. The conjugate is mainly found at the cell surface in CT26.3E10 and in the positive control SKRC-52. No cell surface binding can be detected for the negative control CT26.wt. Red = AAZ-AlexaFluor594 staining; Blue = Hoechst 33342 staining; Scale bar = 100 µm. (D) Evaluation of the in vivo targeting performance of AAZ-IRdye680RD (4) in immunocompetent BALB/c mice bearing CAIX transfected CT26.3E10 tumors by near-infrared fluorescence imaging. A selective tumor uptake at early time points (3 and 6 hours) was observed, in comparison to the biodistribution of the molecule in CAIX-negative CT26.wt tumor bearing mice.

After antibiotic selection and single cell sorting, monoclonal cell lines were tested for CAIX expression. Clone CT26.3E10 (but not CT26.wt) showed a shift in fluorescence intensity in FACS analysis using an anti-CAIX monoclonal antibody. The FACS shift was similar to the one observed for SKRC-52 cells (figure V.13B). Fluorescence microscopy experiments confirmed CAIX expression on the membrane of CT26.3E10 and SKRC-52 cells, but not in wild type CT26 cells (figure V.13C and IV.13D). Furthermore, mice bearing CT26.3E10 tumors showed a preferential uptake of the near-infrared fluorophore conjugate AAZ-IRdye680RD in the neoplastic mass 6 hours after intravenous injection, compared to similar experiments performed in mice bearing CT26.wt tumors (figure V.13E).

Therapy experiments were then performed using compound 2, alone or in combination with the immunocytokine L19-IL2, in immunocompetent BALB/c mice bearing subcutaneous CT26.3E10 tumors.
Figure V.14 Therapeutic activity of AAZ\(^{-}\)-ValCit-MMAE in combination with the immunocytokine L19-IL2 in BALB/c mice bearing CT26.3E10 colorectal carcinoma. BALB/c mice bearing established subcutaneous, human CAIX expressing tumors were treated with AAZ\(^{-}\)-ValCit-MMAE (compound 2; 250 nmol/kg; IV; black arrow) and L19-IL2 (2.5 mg/Kg; IV; white arrow), as monotherapy or in a combination regimen. Graph (A) compares the therapeutic activity of the different treatments. Data points represent mean tumor volume ± SEM, n = 5 per group. CR = Complete Responses. In graph (B) percentage changes of body weight during the experiment are represented. **** indicates p<0.0001; *** indicates p<0.001; ** indicates p<0.01 (2-way ANOVA test, followed by Bonferroni post-test).

Also in this case, the anti-tumor effect was strongest when both therapeutic agents were used in combination (p<0.0001), leading to durable complete responses in 100% of treated mice (figures IV.14 and IV.15). After the therapy study, cured mice were re-challenged by a second subcutaneous injection of CT26.3E10 cancer cells and tumors regrew in all cases. The observation that the combination treatment was not able to confer protective immunity to the treated animals is in keeping with the predominant role played by NK cells, following L19-IL2 accumulation in the neoplastic mass [202].
Day 13 after tumor implantation

Vehicle

L19IL2 (2.5 mg/Kg)

AAZ⁺ValCit-MMAE (250 nmol/Kg)

AAZ⁺ValCit-MMAE (250 nmol/Kg) + L19IL2 (2.5 mg/Kg)

**Figure V.15** Photos of BALB/c immunocompetent mice bearing subcutaneous CT26.3E10 tumors, on day 13 after tumor implantation, after having received different treatments.
3. Discussion

An acetazolamide derivative (named AAZ\textsuperscript{+}), which recognizes CAIX with dissociation constant in the subnanomolar range, was coupled to the linker-payload combination of Adcetris\textsuperscript{TM} (an approved ADC product), featuring the valine-citrulline dipeptide, a self-immolative linker and the anti-tubulin agent MMAE. The new SMDC product mediated a potent tumor growth inhibition activity, but was not able to induce durable complete remissions, when used as single agent in tumor-bearing mice. However, the same product, used in combination with L19-IL2, mediated complete cancer eradication in all nude mice with xenografted SKRC-52 renal cell carcinomas, as well as in all immunocompetent mice bearing a syngeneic CT26 colorectal cancer, transfected with human CAIX.

L19-IL2 is a fully-human antibody-cytokine fusion protein [202], which has been extensively studied in preclinical models of cancer and in clinical trials in patients with various types of malignancies [203, 206]. Encouraged by the results observed in Phase II studies [204, 205], the product is currently being investigated in a Phase III clinical trial as combination agent for the treatment of resectable Stage III B,C melanoma (NCT02938299), which is authorized in the U.S.A., Germany, Italy and Poland.

From a mechanistic viewpoint, the antibody-based delivery of interleukin-2 to the tumor environment promotes the influx of leukocytes into the neoplastic mass [128, 202, 207] and an increase in vascular permeability at the tumor site [128, 208], favoring a preferential uptake of other therapeutic agents used in combination. The observation that L19-IL2 displays a similar activity in nude mice and in immunocompetent mice bearing the same murine tumor suggests that, at least in mice, the product mediates an anti-cancer effect by inducing the migration, proliferation and activation of NK cells [202, 207]. The use of tumor-targeting antibody fusions as an avenue to increase the therapeutic index
of interleukin-2 has been described also in reports published by other groups [209, 210].

The targeted delivery of interleukin-2 to the sub-endothelial extracellular matrix in solid tumors and in certain hematological malignancies has been shown to synergize with other therapeutic modalities, such as the use of chemotherapy [128], radiotherapy [211], intact antibodies capable of triggering an Antibody-Dependent Cell Cytotoxicity (ADCC) [207], as well as various forms of armed antibody derivatives. A synergistic therapeutic activity has been reported for the combination of IL2-based immunocytokines with other antibody-cytokine fusion proteins (e.g., those based on TNF or on interleukin-12) [130, 212] and for ADC products [160].

CAIX has initially been assumed to be an internalizing antigen, suitable for the intracellular delivery and release of potent cytotoxic payloads coupled to CAIX-specific antibodies [84]. However, subsequent studies have clearly shown that CAIX does not efficiently internalize, at least when bound to small molecule ligands [49, 132]. The targeted delivery of linker-payload combinations to the extracellular environment is attractive, as it may promote an efficient drug release process, triggered by high local concentration of glutathione (released by dying tumor cells) [33, 49] or by proteases [103, 163]. Potent cytotoxic drugs may diffuse into the tumor mass, thus potentially acting also on neighboring tumor cells, endothelial cells and/or tumor-resident leukocytes.

The combined use of L19-IL2 with the SMDC product 2 (AAZ⁺-ValCit-MMAE) leads to cancer eradication in two mouse models, which do not respond to conventional chemotherapy or to other experimental therapeutic strategies [49]. Since the corresponding targets (i.e., the alternatively-spliced EDB domain of fibronectin and CAIX) are strongly expressed in the majority of patients with clear cell renal cell carcinoma and in various types of other malignancies (including colorectal cancer), the preclinical results presented in this study provide a rationale for the
clinical investigation of this combination modality. A companion diagnostic, featuring the use of acetazolamide derivatives labeled with technetium-99m, has exhibited promising results in biodistribution studies and has recently received authorization to start clinical studies, with SPECT imaging of cancer patients (EUDRACT n° 2016-004909-13). The study will be important, in order to elucidate whether similar tumor:organ and tumor:blood ratios can be observed both in mice and in patients. Ideally, the SMDC + L19-IL2 combination should be administered to those subjects, who show an adequate uptake of acetazolamide derivatives in the neoplastic masses, using Nuclear Medicine procedures [50].
VI. CHEMICALLY-DEFINED ANTIBODY- AND SMALL MOLECULE-DRUG CONJUGATES FOR IN VIVO TARGETING APPLICATIONS: A COMPARATIVE ANALYSIS


1. Introduction

Conventional cancer chemotherapeutic agents do not preferentially localize at the tumor site [22, 133-135]. This pharmacokinetic limitation often contributes to the onset of toxicity and prevents dose escalation to therapeutically-active regimens [193]. 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) [10, 27, 141] and small molecule-drug conjugates (SMDCs) [9]. Four ADC products have gained marketing authorization for cancer therapy [38, 41, 194-196], while SMDCs are still under investigation in clinical trials [8].

Monoclonal antibodies can recognize their molecular target with exquisite specificity, but their penetration into solid tumor masses can be suboptimal [44]. ADCs have high cost-of-goods and their long circulatory half-life may cause premature drug release [213, 214]. While antibodies can be routinely generated against virtually any protein antigen [56], 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) [47, 49, 66, 148, 149].
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 over-expressed 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 [66, 178]. CAIX is a non-internalizing antigen [49] which has been targeted in clinical trials with radionuclide conjugates [82, 83] and with an ADC product [84]. 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 proteolytic events or by the reduction of disulfide linkers [33, 49, 87, 96, 103].

2. Results

2.1 ADCs and SMDCs generation and in vitro characterization

For the development of an ADC product against CAIX, we used a high-affinity monoclonal antibody, isolated from a phage display library. The SMDC product was based on an acetazolamide derivative with sub-nanomolar dissociation constant to CAIX, recently isolated from a DNA-encoded chemical library [63]. For both agents, we used the linker-payload of Adcetris™, an approved ADC product [215, 216], featuring a cleavable Val-Cit dipeptide, a self-immolating spacer and monomethyl auristatin E (MMAE) as cytotoxic drug (figure VI.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 [34, 217] (figure VI.1). The SMDCs featured a drug-ligand ratio (DLR) of 1. 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 VI.1).
Figure VI.1 Chemical structures and biochemical characterization of anti-CAIX ADC and SMDC products. Ligand-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.
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 VI.1).

2.2 Comparison of the tumor-targeting properties of anti-CAIX ligands

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 ratio 48 hours after the injection, while the small organic ligand showed a substantially higher tumor uptake (~40% ID/g) and a tumor:blood ratio of ~100:1 (figure VI.2A, V.3 and V.4). 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 VI.2B and V.2C). 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 [44], 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 VI.2, V.3 and V.4).
Figure VI.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.
Figure VI.3 Organ distribution of radiolabeled SM(+)\textsuperscript{99m}Tc chelator in BALB/c nu/nu mice bearing SKRC-52 xenografts (n = 3). The data, expressed as mean % Injected Dose/gram of tissue ± SD (%ID/g), correspond to the 6 hours time point after the intravenous administration of the radiolabeled preparation of the ligand.

Figure VI.4 Organ distribution of radiiodinated IgG1(XE114) in BALB/c nu/nu mice bearing SKRC-52 xenografts (n = 5). The data, expressed as mean % Injected Dose/gram of tissue ± SD (%ID/g), correspond to the 48 hours time point after the intravenous administration of the radiolabeled preparation of the antibody.
2.3 Therapeutic comparison between ADCs and SMDCs in the SKRC-52 renal cell carcinoma model

Therapy experiments were performed in nude mice bearing subcutaneously-grafted human SKRC-52 kidney cancer xenografts at equimolar doses of cytotoxic agent. 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 VI.5). 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. 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 VI.5 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.
3. Discussion

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 [44, 218, 219]. 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.

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 VI.2. It is likely that the anti-CAIX ADC product displays its activity, at least in part, 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.

It is possible that the SMDC agent may benefit from a careful tuning of the velocity of payload release [163]. Considering that the high-affinity acetazolamide-based CAIX ligand described in this article displays a tumor:blood 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 [54, 55]. 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.
VII. DISCUSSION, CONCLUSION AND OUTLOOK

1. Discussion
We have shown that acetazolamide, a small organic ligand of carbonic anhydrase IX, can efficiently deliver various types of payload (dyes, radioactive metal ions and cytotoxic drugs) to antigen-positive tumors. We have also shown by confocal laser microscopy that acetazolamide does not efficiently internalize in CAIX-positive cancer cells, but rather accumulate on the cell membrane. Quantitative biodistribution experiments with radiolabeled acetazolamide derivatives have revealed a preferential tumor accumulation of this class of compounds, with tumor:blood ratios of about 80:1 already six hours after intravenous administration. The tumor uptake, as well as the accumulation in some healthy organs like stomach, lung and intestine, was progressively reduced at the highest dose of radiotracer (i.e., above 250 nmol/Kg), this defining an upper limit for target saturation and for in vivo pharmacodelivery applications. The in vivo characterization of the pharmacokinetic properties of acetazolamide derivatives has guided us in the design of a novel set of acetazolamide-based small molecule-drug conjugates, facilitating the choice of dose and schedules for these products.

Acetazolamide was conjugated to two highly-potent cytotoxic drugs, MMAE and PNU-159682, through a valine-citrulline dipeptide linker. The serum stability of the new SMDCs (6-7 hours) is compatible with the fast tumor accumulation and the short circulation time of the targeting moiety. By drug conjugation to acetazolamide, we generated pro-drugs with reduced potency compared to the corresponding free drug payloads, as shown by in vitro cytotoxic assays on CAIX-positive SKRC-52 renal cell carcinoma cells. The experimentally observed cytotoxic profile of acetazolamide-drug conjugates was expected for non-internalizing SMDC products, while an in vitro potentiation of cytotoxic activity compared to
the free drug would have been expected for a ligand, capable of selective internalization into antigen-positive cells [35, 220-222].

Therapy experiments have shown that AAZ-ValCit-MMAE and AAZ-ValCitr-PNU-159682 exhibited a strong anti-tumor activity against SKRC-52 renal cell carcinoma subcutaneously grafted in immunodeficient mice. Importantly, products in which acetazolamide was replaced with an irrelevant amide did not show any detectable anti-cancer effect at equimolar doses, confirming the essential requirement of CAIX recognition for potent therapeutic activity. These results, together with other recent data produced by our group [34, 103, 104], indicate that the valine-citrulline linker, a structure commonly exploited for the development of internalizing ADCs, may also be suitable for the generation of efficacious non-internalizing SMDC and ADC products. The broadly accepted postulate, according to which the valine-citrulline dipeptide would be a specific substrate for the intracellular protease cathepsin B, has been recently questioned [105, 106]. Indeed, other internalizing ADCs based on the valine-citrulline linker were found to be potently active even when the expression of cathepsin B was down-regulated [106].

In Chapter IV of this Dissertation I have shown that that a fine-tuning of the drug release rate in SMDC prodrugs may be crucially important for therapeutic performance. We compared four AAZ-based anti-CAIX SMDCs, differing only for a single amino acid in the cleavable linker structure. By substitution of the valine-citrulline linker in AAZ-ValCit-MMAE with other three structures (valine-alanine, valine-lysine and valine-arginine) we could increase the drug release rate, as revealed by serum stability assays with mass spectrometric detection. The short serum half-life of the valine-arginine linker structure was associated with the lowest in vivo anti-cancer activity against SKRC-52 renal cell carcinomas xenografted in nude mice. A rapid release of the MMAE cytotoxic payload in vivo may contribute to off-target toxicity and may limit therapeutic activity. Interestingly, the most serum-stable structures valine-
citrulline and valine-alanine containing SMDCs were identified as the best performing pro-drugs in terms tolerability and anti-cancer activity in vivo. However, in spite of their efficacy, none of the SMDCs were able to completely eradicate xenografted renal cell carcinomas, when used as single agents at doses below CAIX saturation (i.e., at 250 nmol/Kg). The observation that a second cycle of injections did not result in any evident tumor growth retardation may indicate the onset of a resistance process in the tumor. However, this was not related with antigen downregulation in vivo, as revealed by CAIX staining of tumors excised from the SMDC-treated mice and by in vivo imaging of the animals.

The substitution of acetazolamide with its high-affinity derivative AAZ\(^+\) allowed the generation of a more efficacious SMDC product, as shown by the direct comparison between AAZ-ValCit-MMAE and AAZ\(^+\)-ValCit-MMAE in therapy experiments presented in Chapter V. The combination of AAZ\(^+\)-ValCit-MMAE with the clinical-stage antibody-cytokine fusion protein L19-IL2 led to complete cures in 100% of the treated animals, both in nude and in immunocompetent tumor-bearing mice. We complemented these studies with a panel of staining experiments, performed on SKRC-52 tumors excised from mice treated with AAZ\(^+\)-ValCit-MMAE or with L19-IL2, alone or in combination. We observed a reduction in viable cancer cells, and an increase in apoptosis and in the presence of infiltrating NK cells, as a result of SMDC pharmacological combination treatment. These findings suggest that the targeted delivery of MMAE may cause an immunogenic cell death, which synergizes with the action of IL2, capable of boosting the ability of natural killer cells to recognize stress proteins on the malignant cells. The dominant role of the innate immune system in the cancer eradication driven by SMDC/L19-IL2 was confirmed by the lack of induction of an immunological memory in immunocompetent mice, which had been cured by the combination treatment. The combination of targeted cytotoxic agents (i.e., ADCs and SMDCs) with immune-mediated cancer therapies (e.g., check-point inhibitors and antibody-cytokine fusion proteins) has previously been proposed as a promising strategy to extend the duration of anti-cancer
responses in patients [186]. Our findings are in previous reports on the in vivo activation and maturation of antigen presenting cells (i.e., dendritic cells) after treatment with ADC products based on MMAE [223, 224].

Over the last decade, SMDCs have been proposed as a possible alternative to ADC products. However, to the best of our knowledge, the work presented in Chapter VI represents the first direct comparison of the two technologies. In order to fairly evaluate the two classes of targeted cytotoxic drugs, we have generated chemically-defined ADC and SMDC products against the same target (CAIX). The study of the in vivo distribution of the ADC product revealed an inefficient penetration of the antibody into the solid tumor mass, while the SMDC showed a rapid and homogeneous tumor uptake. Both products revealed a potent in vivo anti-cancer activity against SKRC-52 tumors. Importantly, we included in this therapy experiment ADC and SMDC negative controls, in which the targeting moieties (the antibody or the small ligand) had been replaced by molecular counterparts of the same size but devoid of any CAIX-binding specificity. While the non-targeted (i.e., negative control) SMDC product was found to be inactive, only a small difference in therapeutic activity was observed between the CAIX-targeting ADC product and its negative control counterpart. It is likely that the potent anti-tumor effect observed with the negative control ADC product (specific to hen egg lysozyme) may be due to a slow release of the cytotoxic payload in circulation.

2. Conclusion and Outlook

In conclusion, a novel set of non-internalizing anti-CAIX small molecule-drug conjugates have been synthesized and characterized, both in vitro and in vivo. A potent anti-cancer effect was observed in nude mice bearing renal cell carcinoma xenografts and in immunocompetent mice grafted with CAIX-expressing colorectal tumors, when the products were given as monotherapy or in combination with the clinical-stage immunocytokine L19-IL2.
Based on these promising preclinical results obtained, we believe that the drug delivery strategy based on small ligands of CAIX may deserve an industrial development for the treatment of patients with CAIX-positive tumors. At this moment in time, we do not know whether the favorable tumor-targeting performance, observed for acetazolamide derivatives in tumor bearing mice, may be translated from rodents to patients. The companion diagnostic PHC-102, a derivative of acetazolamide radiolabeled with technetium-99m, will be soon studied in clinical trials (EUDRACT n° 2016-004909-13) by SPECT imaging of patients with clear-cell renal cell carcinomas. If positive, results from those imaging studies may validate CAIX as a suitable target for SMDC development and may provide an experimental methodology, for the selection of suitable patients for therapeutic intervention.

While ADC products have been studied for more than two decades, only a handful of SMDC products has been characterized so far. In most cases, ligands against folate receptor, PSMA, somatostatin receptor or CAIX were used, highlighting the need for high-affinity small organic ligands as an indispensable requirement for pharmacodelivery applications. Monoclonal antibodies can easily be generated against virtually every molecular target of interest, but the discovery of high-affinity small organic ligands to protein targets is more difficult. The field of DNA-encoded chemical libraries allows the construction and screening of compound collections of unprecedented size, thus facilitating hit discovery [57-62]. We anticipate that the lessons learned with CAIX-targeting acetazolamide derivatives may be extended to other molecular targets, as soon as high-affinity ligands become available. In many cases, the in vivo validation of monoclonal antibodies with suitable tumor targeting characteristics will provide a rationale for the choice of suitable targets, for the discovery of small organic ligands and for the generation of novel SMDC products.
VIII. APPENDIX I

ACETAZOLAMIDE SERVES AS SELECTIVE DELIVERY VEHICLE FOR DIPEPTIDE-LINKED DRUGS TO RENAL CELL CARCINOMA – EXPERIMENTAL PROCEDURES

This section corresponds to the experimental part of our journal article entitled “Acetazolamide serves as selective delivery vehicle for dipeptide-linked drugs to renal cell carcinoma” published in Molecular Cancer Therapeutics in 2016 [132].

1. Biological Procedures

1.1 Cell Cultures

The human renal cell carcinoma cell line SKRC-52 was kindly provided by Professor E. Oosterwijk in 2008 (Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands) and subsequently stored in liquid nitrogen. Upon thawing, cells were kept in culture for no longer than 14 passages, tested for CAIX expression by immunofluorescence and not further authenticated. SKRC-52 and HEK293 (ATCC, CRL-1573) were maintained in RPMI medium (Invitrogen) supplemented with fetal calf serum (10%, FCS, Invitrogen) and Antibiotic-Antimycotic (1%, AA, Invitrogen) at 37 °C and 5% CO₂. For passaging, cells were detached using Trypsin-EDTA 0.05% (Invitrogen) when reaching 90% confluence and re-seeded at a dilution of 1:6.

1.2 Expression and Purification of Recombinant Human CAIX

Carbonic anhydrase IX (CAIX) was cloned in pCEP4 with a secretion sequence at its 5’, and expressed in HEK293-ebna cells by transient gene expression. The transfection mix was assembled as follow: 1.25 µg of plasmid DNA, 3.75 µg polyethylenimine (PEI) for 10⁶ cells, with a cell
density of $20 \times 10^6$ cells/mL for 3 hours at 37 °C, 5% CO$_2$, 120 rpm. After high-density incubation, cells were diluted to a concentration of $10^6$ cells/mL and incubated for 6 days at 37 °C, 5% CO$_2$, 120 rpm. Cells were harvested by centrifugation (4500 rpm 30 min, 4 °C), and 1 mL (dry volume) of cOmplete His-tag purification Resin (Roche) was added to the filtered supernatant and incubated for 2 hours, 120 rpm at room temperature. Resin was washed with 300 mL of wash-buffer (imidazole 10mM, PBS/NaCl 250mM) and CAIX was eluted with 250 mM imidazole PBS/NaCl 250 mM. Elution fractions (1 mL) were loaded onto SDS-Page. CAIX-enriched fractions were pooled and dialyzed against PBS.

1.3 Surface Plasmon Resonance
Surface Plasmon Resonance (SPR) experiments were performed on a Biacore S200 instrument (GE Healthcare) at room temperature. For all measurements, CM5 chips (Series S) and flow buffer of filtered PBS pH 7.4 with DMSO (5% v/v) were used. Human CAIX was immobilized on the chip to 500 response units (RU) using EDC·HCl and NHS following manufacturer instructions. Serial dilutions of compounds 3a and 3b in running buffer at a flow rate of 20 µL min$^{-1}$ were used as analytes. After each cycle, the sensor surface was regenerated by a short treatment with DMSO (50% v/v) in PBS. Sensorgrams were solvent corrected and the binding kinetics were analyzed with the Biacore S200 evaluation software using a 1:1 Langmuir.

1.4 Ligand Internalization Analysis by Confocal Microscopy
SKRC-52 or HEK 293 cells were seeded into 4-well cover slip chamber plates (Sarstedt) at a density of $10^4$ cells per well in RPMI medium (1 mL, Invitrogen) supplemented with 10% FCS, AA and HEPES (10 mM) and allowed to grow for 24 hours under standard culture conditions. The medium was replaced with medium containing 2a or 2b (120 nM), after 30 min or 1 hour Hoechst 33342 nuclear dye (Invitrogen) was added and
randomly selected colonies imaged on a SP8 confocal microscope equipped with an AOBS device (Leica Microsystems).

1.5 Linker Stability Assays
Compounds 4a and 5a were dissolved in PBS (100 µg/mL) and incubated at 37 °C in a shaking incubator. Aliquots (150 µL) were taken at different time points (0, 15’, 1h, 6h, 24h, 48h) and frozen at -20°C. Standard solutions of the conjugates were prepared in PBS at different concentration (25, 50, 100, 150 µg/mL) and stored at -20 °C. The standards and the samples were analyzed in triplicate by Liquid Chromatography - Mass Spectrometry (LC-MS) on a Waters Xevo G2-XS QTOF coupled to a Waters Acquity UPLC H-Class System using a ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm column. Peaks relative to the intact SMDCs were integrated and area values from the standards were used to obtain calibration curves. Concentration of intact test compounds in the samples was determined at the different time points to obtain a stability profile of the conjugates.

To measure mouse serum stabilities of the conjugates, the compounds were dissolved in freshly thawed mouse serum (Invitrogen) (100 µg/mL) and incubated at 37 °C in a shaking incubator. Aliquots of 150 µL were taken at different time points (0, 20 min, 1, 3, 6 and 24 hours) and frozen at -20 °C. Standard solutions were prepared in mouse serum (Invitrogen) at different concentration (25, 50, 100, 150 µg/mL) and stored at -20 °C. All the samples and the standards were thawed and diluted with four volumes of MeOH. After vigorous vortex agitation for 1 min, the protein precipitate was spun down and 400 µL of the supernatant was lyophilized overnight. The resulting solid material was re-dissolved in 100 µL of Millipore water and analyzed as described above.

1.6 In Vitro Cytotoxicity Assay
SKRC-52 cells were seeded in 96-well plates in RPMI added with 10% FCS (100 µL) at a density of 5000 cells per well and allowed to grow for
24 hours. The medium was replaced with medium containing different concentrations of test substance (1:3 dilution steps) and plates were incubated under standard culture conditions. After 72 hours the medium was removed, MTS cell viability dye (20 µL, Promega) in 150 µL of the medium was added, the plates were incubated for 2 hours under culture conditions and the absorbance at 490 nm measured on a Spectra Max Paradigm multimode plate reader (Molecular Devices). Experiments were performed in triplicate and average cell viability calculated as measured background corrected absorbance divided by the absorbance of untreated control wells. IC$_{50}$ values were determined by fitting data to the four-parameter logistic equation, using a Prism 6 software (GraphPad Software) for data analysis.

1.7 Animal Studies

All animal experiments were conducted in accordance with Swiss animal welfare laws and regulations under the license number 27/2015 granted by the Veterinäramt des Kantons Zürich.

1.8 Implantation of Subcutaneous SKRC-52 Tumors

SKRC-52 cells were grown to 80% confluence and detached with Trypsin-EDTA 0.05% (Life Technologies). Cells were washed with Hank’s Balanced Salt Solution (HBSS, pH 7.4) once, counted and re-suspended in HBSS to a final concentration of 3.4 × 10$^7$ cells mL$^{-1}$. Aliquots of 5 × 10$^6$ cells (150 µL of a suspension) were injected subcutaneously in the right flank of female athymic BALB/c nu/nu mice (8–10 weeks of age, Janvier).

1.9 Quantitative Biodistribution Studies

SKRC-52 xenografted tumors were implanted into female BALB/c nu/nu mice (Janvier) as described above, and allowed to grow for three weeks to an average volume of 0.25 mL. Mice were randomized (n = 5 per group) and injected intravenously with different doses of radiolabelled
preparations (15-25 MBq, 70-560 nmol/Kg). Mice were sacrificed 6 hours after the injection by CO₂ asphyxiation and organs extracted, weighted and radioactivity measured with a Packard Cobra γ-counter. Values are expressed as %ID/g ± SD.

1.10 IVIS Imaging
Female BALB/c nu/nu mice bearing subcutaneous SKRC-52 tumors were injected intravenously with Compound 3a, containing the near infrared dye moiety IRDye680RD (LI-COR Biosciences) (250 nmol/Kg), dissolved in sterile PBS (100 µL). Mice were anesthetized with isoflurane and fluorescence images acquired on an IVIS Spectrum imaging system (Xenogen, exposure 1s, binning factor 8, excitation at 675 nm, emission filter at 720 nm, f number 2, field of view 13.1). Images were taken before the injection and after 10 min, 1, 3 and 6 hours. Food and water was given ad libitum during that period.

1.11 Dose Escalation Study
Recommended dose of compound 4a suitable for therapy experiments was determined by dose escalation in wild type female athymic BALB/c nu/nu mice (8–10 weeks of age, Janvier). A schedule of five injections on five consecutive days was used to compare increasing doses (250 nmol/Kg or 500 nmol/Kg) of the targeted derivative 4a with untargeted compound 4b (n = 3). Tolerated dose was defined when animals did not lose more than 5% of their initial body weight over the duration of the experiment after the initial injection. Recomended dose of compound 5a was determined in the same experimental way (n = 1).

1.12 Therapy Experiments
SKRC-52 xenografted tumors were implanted into female BALB/c nu/nu mice (Janvier) as described above, and allowed to grow for two weeks to an average volume of 0.1 mL. Mice were randomly assigned into therapy groups of 4 or 5 animals and treatment started by injecting a solution of
the targeted drugs, untargeted drugs or vehicle (PBS only or PBS containing 1% of DMSO) intravenously (tail vein) at the doses and with the schedules indicated in the text. Compounds 4a,b were injected as solutions in sterile PBS. Compounds 5a,b were injected as solutions in sterile PBS containing 1% DMSO. Animals were weighed and tumor sizes measured daily with an electronic caliper. The tumor volume was calculated according to the formula (long side) × (short side) × (short side) × 0.5. Animals were sacrificed when the termination criteria were reached. Prism 6 software (GraphPad Software) was used for data analysis (regular two-way ANOVA with the Bonferroni test).

1.13 Immunofluorescence Studies
SKRC-52 tumors were excised from the animals treated with conjugate 5a or alternatively with vehicle during the therapy experiment, embedded in OCT medium (Thermo Scientific), and cryostat sections (10 µm) were cut. Slides were stained using the following antibodies: mouse anti-human CAIX GT12 (Thermo Scientific), to detect the antigen, and rat anti-mouse CD31 (BD Biosciences) to detect endothelial cells. Nuclear staining was performed with DAPI. Anti-mouse 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.

2. Chemical Procedures
2.1 General Chemical Procedures
Anhydrous solvents for reactions were purchased from Acros or Fluka. Peptide grade N,N-dimethylformamide (DMF) for solid phase synthesis was bought from ABCR. All other solvents were used as supplied by Fisher Chemicals, Merck or Sigma Aldrich in HPLC or analytical grade. Fmoc-Cys(Trt)-OH Polystyrene Resin was purchased from RAPP Polymere. Ultra-Technekow DTE Mo-99/Tc-99m Generator was purchased from Mallinckrodt Pharmaceuticals. Maleimidocaproyl-
Alexa594 was purchased from ThermoFisher Scientific. Maleimidoethyl-IRDye680RD was purchased from LI-COR Biosciences. Maleimidoacryloyl-ValCit-p-aminobenzylalcohol-MMAE, Maleimidoacryloyl-PEG(4)-ValCit-p-aminobenzylalcohol-DMEDA-PNU159682 and corresponding free drugs were purchased from Levena Biopharma. All other reagents were purchased from Sigma Aldrich, Acros, ABCR or TCI and used as supplied. All reactions using anhydrous conditions were performed using oven-dried glassware under an atmosphere of argon. Silica for flash column chromatography (high-purity grade, pore size 60 Å) was purchased from Sigma Aldrich. Reactions were monitored by thin layer chromatography using Merck silica gel 60 F254 TLC glass plates. Concentration under reduced pressure was performed by rotary evaporation at 40 °C at the appropriate pressure. Yields refer to chromatographically purified and spectroscopically pure compounds, unless noted otherwise.

Proton (1H) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV400 (400 MHz) or a Bruker AVIII500 (500 MHz) spectrometer. Shifts are given in ppm using residual solvent as the internal standard. Coupling constants (J) are reported in Hz with the following abbreviations used to indicate splitting: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet.

High-Resolution Mass Spectrometry (HR-MS) spectra and analytical Reversed-Phase Ultra Performance Liquid Chromatography (UPLC) were recorded on a Waters Xevo G2-XS QTOF coupled to a Waters Acquity UPLC H-Class System with PDA UV detector, using a ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 μm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min\(^{-1}\) with linear gradients of solvents A and B (A = Millipore water with 0.1% formic acid [FA], B = MeCN with 0.1% formic acid [FA]). Calculated and exact m/z values are reported in Daltons.

Analytical and preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) were performed on a Waters Alliance HT RP-HPLC with PDA UV detector, using a Synergi 4μm, Polar-RP 80Å 10 × 150 mm C18 column at a flow rate of 4 mL min\(^{-1}\) with linear gradients of solvents A and B (A = Millipore water with 0.1% trifluoroacetic acid [TFA],
B = MeCN with 0.1% trifluoroacetic acid [TFA]). Alternatively a Waters Xterra 5 µm 150 Å 10 × 150 mm C18 column at a flow rate of 2.8 mL min⁻¹ was used with the same eluent phase.

2.2 Peptide Synthesis

Peptidic precursors of compounds 1-5 were synthesized by solid phase peptide synthesis (SPPS), using Fmoc-protected amino acids. Chlorotrityl resin (500 mg), pre-loaded with Fmoc-Cys(Trt)-OH, was swollen with DMF (10 mL) for 15 min inside a syringe equipped with a filter pad. Fmoc deprotection was achieved by shaking the resin with 20% v/v piperidine in DMF (5 mL) for 10 min for three times. After deprotection, the resin was washed with DMF (4 × 10 mL). Fmoc-protected amino acids (3 eq) were activated with HATU (3 eq) and DIPEA (6 eq) in DMF (5 mL) for 15 min in ice bath. After this time the solution was allowed to react with the syringe for 1 hour, discarded and the resin washed with DMF (4 × 10 mL). Coupling and deprotection steps were alternated, in order to obtain the desired peptide sequence. Acetazolamide or free amide moieties were then introduced by performing a CuAAC “click” reaction [225] with the corresponding peptide derivatives (carrying an azide moiety) on solid phase, followed by side chain deprotection, cleavage from the resin and RP-HPLC purification.

2.3 Radiolabelling

Radiolabeling procedures with technetium-99m were performed essentially as described [88]. Briefly, compound 1 (60 nmol) in TBS pH 7.4 (50 µl) was mixed with SnCl₂ (Sigma Aldrich, 200 µg) and sodium glucoheptonate (TCI, 20 mg) in H₂O (150 µl). Tris-buffered saline at pH 7.4 (600 µl) was added and the resulting solution degassed for 5 min by bubbling with nitrogen gas. The eluate from a ⁹⁹ᵐ⁻Tc-generator (200 µl, ca. 200 MBq, Mallinckrodt) was added and the reaction mixture heated to 90 °C for 20 min. After cooling to room temperature, an aliquot was analyzed by RP-HPLC (XTerra C18, 5% MeCN in 0.1% aq. TFA to 80% over 20
min on a Merck-Hitachi D-7000 HPLC system equipped with a Raytest Gabi Star radiodetector). Technetium-99m incorporations >95% were routinely achieved. The radioactive solution was then diluted to the desired concentrations with a molar excess of compound 1, dissolved in TBS, pH 7.4.
2.4 Chemical Synthesis of Present Compounds

AAZ-\textsuperscript{\textit{Tc}} \textit{chelator} – 1

\[
\text{Chemical Formula: } C_{30}H_{46}N_{12}O_{13}S_{3} \\
\text{Molecular Weight: 878.95}
\]

Commerically available pre-loaded Fmoc-Cys(Trt)-OH on polystyrene resin (500 mg, 0.32 mmol) was swollen in DMF for 15 min. The Fmoc group was removed with 20% piperidine in DMF (3 \times 10 \text{ min} \times 10 \text{ mL}) and the resin washed with DMF (4 \times 10 \text{ min} \times 10 \text{ mL}). Fmoc-Asp(OtBu)-OH (389 mg, 0.95 mmol, 3 eq) was activated with HATU (359 mg, 0.95 mmol, 3 eq), and DIPEA (329 \mu l, 1.89 mmol, 6 eq) in DMF (5 mL) at 0 °C for 15 min and then reacted with the resin for 1 hour under gentle agitation. After washing the resin with DMF (4 \times 10 \text{ min} \times 10 \text{ mL}) the Fmoc group was removed with 20% piperidine in DMF (3 \times 10 \text{ min} \times 10 \text{ mL}) and the resin washed with DMF (4 \times 10 \text{ min} \times 10 \text{ mL}) before the peptide was extended with N\textsubscript{\alpha}-Boc-Lys(Fmoc)-OH (445 mg, 0.95 mmol, 3 eq), Fmoc-Asp(OtBu)-OH (389 mg, 0.95 mmol, 3 eq) and 5-azido pentanoic acid (136 mg, 0.95 mmol, 3 eq) in the indicated order using the same coupling conditions (HATU/DIPEA), Fmoc-deprotection (20% piperidine in DMF) and washing step with DMF mentioned before. After the last peptide-coupling step, a solution of Cul (18 mg, 0.09 mmol, 0.28 eq), TBTA (16.7 mg, 0.03 mmol, 0.09 eq) and alkyne 9 (260 mg, 1.04 mmol, 3 eq) in a mixture of DMF (2.5 mL) and THF (2.5 mL) was prepared and reacted with the resin at room temperature for 48 h. After washing with DMF (4 \times 10 \text{ min} \times 10 \text{ mL}), EDTA 50 mM (4 \times 10 \text{ min} \times 10 \text{ mL}) and DCM (4 \times 10 \text{ min} \times 10 \text{ mL}), the compound was cleaved from the resin by agitating with a mixture of TFA (8.6 mL), TIS (1.6 mL), H\textsubscript{2}O (400 mL), and stirring for 4-6 h.
µl), m-Cresol (400 µl) and Thioanisol (400 µl) at room temperature for 1 hour. Cleavage solution was added drop-wise to ice cold diethyl ether (50 mL) obtaining a white precipitate. The pellet was collected by centrifugation, dried under vacuum, redissolved in Millipore water and added with an excess of Tris(2-carboxyethyl)phosphine hydrochloride. The product was purified by reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). After lyophilization the final compound was collected as a white powder (70 mg, 79.6 µmol, 25% yield).

\(^1\)H (400 MHz, DMSO-d6) \(\delta \) [ppm] = 12.99 (s, 1H), 12.54 (broad s, 2H), 8.74 (d, \(J = 7.6 \text{ Hz}, 1\)H), 8.32 (s, 2H), 8.25 (d, \(J = 7.8 \text{ Hz}, 1\)H), 8.08 (m, 4H), 7.87 (s, 1H), 7.83 (t, \(J = 5.6 \text{ Hz}, 1\)H), 4.67 (m, 1H), 4.50 (m, 1H), 4.40 (m, 1H), 4.30 (t, \(J = 7.0 \text{ Hz}, 2\)H), 3.73 (s, 1H), 3.01 (m, 3H), 2.72 (m, 10H), 2.41 (m, 2H), 2.16 (t, \(J = 7.4 \text{ Hz}, 2\)H), 1.96 (m, 2H), 1.74 (m, 4H), 1.35 (m, 6H).

HRMS: \((m/z) [M+H]^+ \ C_{30}H_{47}N_{12}O_{13}S_{3}, 878.2469, \text{ found 878.2473.}

Figure VIII.1 Analytical HPLC trace of compound 1 on a Synergi RP Polar column, 5% MeCN in 0.1% aq. TFA to 80% MeCN in 20 min.
To a solution of compound 7 (5.1 mg, 5.6 µmol) in degased PBS (pH 7.4; 1 mL) was added the commercially available Maleimidocaproyl-Alexa594 (0.5 mg, 0.55 µmol) dissolved in DMF (300 µl). The mixture was stirred at room temperature for 3 hours and the solvent was removed under vacuum. The conjugate was purified over RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min) and product containing fractions identified by mass spectrometry and lyophilized overnight to obtain a blue powder (615 µg, 0.35 µmol, 63%).

HRMS: (m/z) [M + 3H]^{2+} C_{74}H_{88}N_{18}O_{25}S_5, 897.2506; found 897.3035.
NH$_2$-Alexa594 (negative control) – 2b

Chemical Formula: C$_{72}$H$_{90}$N$_{15}$O$_{23}$S$_{3}$

Molecular Weight: 1629.77

To a solution of compound 6 (4.1 mg, 5.5 µmol) in degased PBS (pH 7.4; 1 mL) was added the commercially available Maleimidocaproyl-Alexa594 (0.5 mg, 0.55 µmol) dissolved in DMF (300 µl). The mixture was stirred at room temperature for 3 hours and the solvent was removed under vacuum. The conjugate was purified over RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min) and product containing fractions identified by mass spectrometry and lyophilized overnight to obtain a blue powder (495 µg, 0.30 µmol, 55%).

HRMS: (m/z) [M + 3H]$^{2+}$ C$_{72}$H$_{93}$N$_{15}$O$_{23}$S$_{3}$, 815.7751; found 815.8274.

Figure VIII.3 Analytical UPLC trace of targeted conjugate 2b on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min$^{-1}$, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
AAZ-IRDye680RD – 3a

Chemical Formula: C_{75}H_{97}ClN_{19}O_{25}S_{6}^{+}
Molecular Weight: 1892.52

To a solution of compound 7 (2.8 mg, 3.1 µmol) in degased PBS (pH 7.4; 500 µl) was added the commercially available Maleimidoethyl-IRDye680RD (1.8 mg, 1.8 µmol) dissolved in degased PBS (pH 7.4; 500 µl). The mixture was stirred at room temperature for 1 hour and the solvent was removed under vacuum. The conjugate was purified over RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min) and product containing fractions identified by mass spectrometry and lyophilized overnight to obtain a dark purple powder (2.7 mg, 1.4 µmol, 79%).

HRMS: (m/z) [M + H]^{2+} C_{75}H_{98}ClN_{19}O_{25}S_{6}^{+}, 945.7455; found 945.7510.

Figure VIII.4 Analytical UPLC trace of targeted conjugate 3a on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min\(^{-1}\), 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
AAZ-ValCit-MMAE – 4a

Chemical Formula: C$_{98}$H$_{151}$N$_{25}$O$_{28}$S$_3$

Molecular Weight: 2223.61

To a solution of compound 7 (4.5 mg, 5 µmol) in degased PBS (pH 7.4; 1 mL) was added the commercially available Maleimidocaproyl-ValCit-p-aminobenzylalcohol-MMAE (6.5 mg, 4.9 µmol) dissolved in DMF (1 mL). The mixture was stirred at room temperature for 5 min and the solvents were removed under vacuum. The conjugate was purified over RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min) and product containing fractions identified by mass spectrometry and lyophilized overnight to obtain a white solid (7.5 mg, 3.4 µmol, 68%).

HRMS: (m/z) [M + 2H]$^{2+}$ C$_{98}$H$_{153}$N$_{25}$O$_{28}$S$_3$, 1112.0234; found 1112.0237.
Figure VIII.5 Analytical UPLC trace of targeted conjugate 4a on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm x 50 mm at a flow rate of 0.6 mL min\(^{-1}\), 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
To a solution of compound 6 (4.5 mg, 5 µmol) in degased PBS (pH 7.4; 1 mL) was added the commercially available Maleimidocaproyl-ValCit-p-aminobenzylalcohol-MMAE (6.5 mg, 4.9 µmol) dissolved in DMF (1 mL). The mixture was stirred at room temperature for 5 min and the solvents were removed under vacuum. The conjugate was purified over RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min) and product containing fractions identified by mass spectrometry and lyophilized overnight to obtain a white solid (7.5 mg, 3.4 µmol, 68%).

HRMS: \([m/z] [M + 2H]^{2+} \text{C}_{96}\text{H}_{150}\text{N}_{22}\text{O}_{26}\text{S}, \text{1030.5406; found 1030.5401.}\)
Figure VIII.6 Analytical UPLC trace of targeted conjugate 4b on a BEH C18 Column, 130 Å, 1.7 μm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
To a solution of compound 7 (2.3 mg, 2.5 µmol) in degased PBS (pH 7.4; 800 uL) was added the commercially available Maleimidocaproyl-PEG(4)-ValCit-p-aminobenzylalcohol-DMEDA-PNU159682 (3.1 mg, 2 µmol) dissolved in DMF (150 uL). The mixture was stirred at room temperature for 5 min and the solvents were removed under vacuum. The conjugate was purified over RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min) and product containing fractions identified by mass spectrometry and lyophilized overnight to obtain compound 5a as a red solid (2.5 mg, 1.0 µmol, 51% yield).

HRMS: (m/z) [M + 2H]^2+ C_{104}H_{144}N_{24}O_{40}S_{3}, 1233.4567; found 1233.4630.
Figure VIII.7 Analytical HPLC trace of targeted conjugate $5a$ on a Waters Xterra 5 µm 150 Å 10 × 150 mm C18 column at a flow rate of $2.8 \text{ mL min}^{-1}$, 5% MeCN in 0.1% aq. FA to 100% MeCN in 30 min.
NH₂-ValCit-PNU159682 (negative control) – 5b

To a solution of compound 6 (2.0 mg, 2.7 µmol) in degased PBS (pH 7.4; 850 uL) was added the commercially available Maleimidocaproyl-PEG(4)-Val-Cit-p-aminobenzylalcohol-DMEDA-PNU159682 (2.0 mg, 1.3 µmol) dissolved in DMF (150 uL). The mixture was stirred at room temperature for 5 min and the solvents were removed under vacuum. The conjugate was purified over RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min) and product containing fractions identified by mass spectrometry and lyophilized overnight to obtain compound 5b as a red solid (2.8 mg, 1.2 µmol, 94% yield).

HRMS: \( m/z \) [M + 2H]²⁺ \( C_{102}H_{143}N_{21}O_{38}S \), 1151.9812; found 1151.9906.

Chemical Formula: \( C_{102}H_{143}N_{21}O_{38}S \)
Molecular Weight: 2303.44
Figure VIII.8 Analytical HPLC trace of targeted conjugate 5b on a Waters Xterra 5 µm 150 Å 10 × 150 mm C18 column at a flow rate of 2.8 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 100% MeCN in 30 min.
Chemical Formula: C\textsubscript{28}H\textsubscript{45}N\textsubscript{11}O\textsubscript{11}S
Molecular Weight: 743.79

Commerically available pre-loaded Fmoc-Cys(Trt)-OH on polystyrene resin (500 mg, 0.32 mmol) was swollen in DMF for 15 min. The Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 mL) and the resin washed with DMF (4 × 10 min × 10 mL). Fmoc-Asp(OtBu)-OH (389 mg, 0.95 mmol, 3 eq) was activated with HATU (359 mg, 0.95 mmol, 3 eq), and DIPEA (329 µL, 1.89 mmol, 6 eq) in DMF (5 mL) at 0 °C for 15 min and then reacted with the resin for 1 h under gentle agitation. After washing the resin with DMF (4 × 10 min × 10 mL) the Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 mL) and the resin washed with DMF (4 × 10 min × 10 mL) before the peptide was extended with Fmoc-Arg(Pbf)-OH (613 mg, 0.95 mmol, 3 eq), Fmoc-Asp(OtBu)-OH (389 mg, 0.95 mmol, 3 eq) and 5-azido pentanoic acid (136 mg, 0.95 mmol, 3 eq) in the indicated order using the same coupling conditions (HATU/DIPEA), Fmoc-deprotection (20% piperidine in DMF) and washing step with DMF mentioned before. After the last peptide-coupling step, a solution of Cul (18 mg, 0.09 mmol, 0.26 eq), TBTA (16.7 mg, 0.03 mmol, 0.08 eq) and alkyne 10 (105 mg, 0.95 mmol, 3 eq) in a mixture of DMF (2.5 mL) and THF (2.5 mL) was prepared and reacted with the resin at room temperature for 48 h. After washing with DMF (4 × 10 min × 10 mL), EDTA 50 mM (4 × 10 min × 10 mL) and DCM (4 × 10 min × 10 mL), the compound was cleaved from the resin by agitating with a mixture of TFA (8.6 mL), TIS (1.6 mL), H\textsubscript{2}O (400
µL), m-Cresol (400 µL) and Thioanisol (400 µL) at room temperature for 1 hour. Cleavage solution was added drop-wise to ice cold diethyl ether (50 mL) obtaining a white precipitate. The pellet was collected by centrifugation, dried under vacuum, redissolved in Millipore water and added with an excess of Tris(2-carboxyethyl)phosphine hydrochloride. The product was purified by reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). After lyophilization the final compound was collected as a white powder (25 mg, 34 µmol, 11% yield).

^1^H (500 MHz, DMSO-d6) δ [ppm] = 12.53 (broad s, 2H), 8.23 (d, J = 7.4 Hz, 1H), 8.17 (d, J = 7.8 Hz, 1H), 8.12 (d, J = 7.7 Hz, 1H), 7.99 (d, J = 7.7 Hz, 1H), 7.52 (broad s, 1H), 7.28 (s, 1H), 6.75 (s, 1H), 4.56 (m, 2H), 4.43 (m, 1H), 4.3 (t, J = 7.0 Hz, 2H), 4.21 (m, 1H), 3.14 (m, 1H), 3.06 (m, 1H), 2.96 (m, 1H), 2.70 (m, 2H), 2.60 (t, J = 7.7 Hz, 2H), 2.50 (m, superimposed with residual DMSO signal), 2.15 (t, J = 7.4 Hz, 2H), 2.11 (t, J = 7.5 Hz, 2H), 1.79 (m, 5H), 1.47 (m, 5H).

HRMS: (m/z) [M+H]^+ C_{28}H_{46}N_{11}O_{11}S, 744.3021, found 744.3028.

Figure VIII.9 Analytical UPLC trace of targeted compound 6 on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
AAZ-SMDC precursor – 7

Chemical Formula: $\text{C}_{30}\text{H}_{46}\text{N}_{14}\text{O}_{13}\text{S}_3$
Molecular Weight: 906.96

Compound 7 was prepared according to a previously described method [49].

5-amino-1,3,4-thiadiazole-2-sulfonamide – 8

Chemical Formula: $\text{C}_2\text{H}_4\text{N}_4\text{O}_2\text{S}_2$
Molecular Weight: 180.20

Compound 8 was prepared according to a previously described method [226].
**N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)hex-5-ynamide – 9**

![Chemical Structure](image)

Chemical Formula: C₈H₁₀N₄O₃S₂  
Molecular Weight: 274.31

Alkyne 9 was prepared according to a previously described method [49].

**Hex-5-ynamide – 10**

![Chemical Structure](image)

Chemical Formula: C₆H₉NO  
Molecular Weight: 111.14

Alkyne 10 was prepared according to a previously described method [227].
2.5 $^1$H NMR spectra

Compound 1

N06_160912_ANSCO1_1H.1.1.1
pnmr_1H_16 DMSO/400MHz 32

Compound 6

N05SC-MBSC_01_500.1.1.1.fr
3. Statistical analysis of therapy experiment

Differences in tumor volume between therapeutic groups were compared using the two-way ANOVA analysis with Bonferroni post-test of Graphpad Prism 6 (La Jolla, CA, USA).

Therapy experiment 1 – AAZ-ValCit-MMAE against SKRC-52 tumors subcutaneously xenografted in nude mice

Tumor Size (mm$^3$)
Compound 4a (AAZ-ValCit-MMAE) vs. Vehicle:

<table>
<thead>
<tr>
<th>Day</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>21</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>22</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>23</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>24</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>from 25</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Compound 4b (NH$_2$-ValCit-MMAE) vs. Vehicle:
non-significant differences

Compound 4a (AAZ-ValCit-MMAE) vs. Compound 4b (NH$_2$-ValCit-MMAE):

<table>
<thead>
<tr>
<th>Day</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>22</td>
<td>non-significant differences</td>
</tr>
<tr>
<td>23</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>24</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>25</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>26</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>27</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>from 28</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Body Weight Change (%) 

Compound 4a (AAZ-ValCit-MMAE) vs. Vehicle:

day 26  p < 0.01

day 27  p < 0.001

from day 28  p < 0.0001

Compound 4b (NH₂-ValCit-MMAE) vs. Vehicle:

non-significant differences

Compound 4a (AAZ-ValCit-MMAE) vs. Compound 4b (NH₂-ValCit-MMAE):

day 27  p < 0.05

from day 28  p < 0.001

Therapy experiment 2 – AAZ-ValCit-PNU-159682 against SKRC-52 tumors subcutaneously xenografted in nude mice

Tumor Size (mm³)

Compound 5a (AAZ-ValCit-PNU-159682) vs. Vehicle:

day 22  p < 0.05

day 23  p < 0.01

day 24  p < 0.001

day 25  p < 0.001

from day 26  p < 0.0001

Compound 5b (NH₂-ValCit-PNU-159682) vs. Vehicle:

non-significant differences

Compound 5a (AAZ-ValCit-PNU-159682) vs. Compound 5b (NH₂-ValCit-PNU-159682):

day 25  p < 0.05

day 26  p < 0.001

day 27  p < 0.001

from day 28  p < 0.0001
Compound 5a (AAZ-ValCit-PNU-159682) vs. Competition group:

day 26  p < 0.05

day 27  non-significant differences

day 28  p < 0.01

day 29  p < 0.01

from day 30  p < 0.0001

Vehicle vs. Competition group:

day 22  p < 0.05

day 23  non-significant differences

day 24  p < 0.01

day 25  p < 0.01

day 26  p < 0.01

from day 27  p < 0.0001

Compound 5b (NH$_2$-ValCit-PNU-159682) vs. Competition group:

day 24  p < 0.05

day 25  p < 0.05

day 26  p < 0.001

from day 27  p < 0.0001

**Body Weight Change (%)**

**Compound 5a (AAZ-ValCit-PNU-159682) vs. Vehicle:**

day 22  p < 0.01

day 23  p < 0.05

day 24  p < 0.05

day 25  p < 0.05

day 26  p < 0.05

day 27  p < 0.05

from day 28  non-significant differences
Compound 5b ($NH_2$-ValCit-PNU-159682) vs. Vehicle:
non-significant differences

Compound 5a (AAZ-ValCit-PNU-159682) vs. Compound 5b ($NH_2$-ValCit-PNU-159682):
non-significant differences

Compound 5a (AAZ-ValCit-PNU-159682) vs. Competition group:
day 22 $p < 0.01$
day 23 $p < 0.05$
day 24 $p < 0.01$
day 25 $p < 0.05$
day 26 $p < 0.05$
from day 27 non-significant differences

Vehicle vs. Competition group:
non-significant differences

Compound 5b ($NH_2$-ValCit-PNU-159682) vs. Competition group:
from day 31 $p < 0.05$
IX. APPENDIX II
LINKER STABILITY INFLUENCES THE ANTI-TUMOR ACTIVITY OF ACETAZOLAMIDE-DRUG CONJUGATES FOR THE THERAPY OF RENAL CELL CARCINOMA – EXPERIMENTAL PROCEDURES

This section corresponds to the experimental part of our journal article entitled “Linker stability influences the anti-tumor activity of acetazolamide-drug conjugates for the therapy of renal cell carcinoma” published in the Journal of Controlled Release in 2017 [163].

1. Biological Procedures

1.1 Cell Cultures
The human renal cell carcinoma cell line SKRC-52 was kindly provided by Professor E. Oosterwijk (Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands) and tested for CAIX expression by immunofluorescence. No further authentication was performed. SKRC-52 were maintained in RPMI medium (Invitrogen) supplemented with fetal calf serum (10%, FCS, Invitrogen) and Antibiotic-Antimycotic (1%, AA, Invitrogen) at 37 °C and 5% CO2. For passaging, cells were detached using Trypsin-EDTA 0.05% (Invitrogen) when reaching 90% confluence and re-seeded at a dilution of 1:6.

1.2 Linker Stability Assays
Compounds 1-4 were incubated at 37 °C in a shaking incubator as PBS solutions (pH 7.4; 100 µg/mL). Aliquots (200 µL) were collected at subsequent time points (0, 15 min, 1, 3, 6, 24 and 96 hours) and frozen at -20 °C. The samples were analyzed in triplicate by LC-MS. Peaks relative to the pro-drugs were integrated and area values from samples at
different time points were used to obtain fractions of intact test compounds. Fraction values were plotted to obtain stability profiles. Analogously, test compounds were dissolved in DMSO (10 µL), diluted with freshly thawed mouse serum (Invitrogen) (100 µg/mL) and incubated at 37 °C in a shaking incubator. Aliquots (100 µL) were collected at subsequent time points (0, 20 min, 1, 3, 6, 24 and 48 hours) and frozen at -20 °C. Samples were thawed and diluted with four volumes of MeOH (400 µL). After vigorous vortex agitation for 1 min, the protein precipitate was spun down and 400 µL of the supernatant was lyophilized overnight. The resulting solid material was re-dissolved in 200 µL of water, filtered and analyzed as described above.

1.3 In Vitro Cytotoxicity Assay
SKRC-52 cells were seeded in 96-well plates in RPMI added with 10% FCS (100 µL) at a density of 5000 cells per well and allowed to grow for 24 hours. The medium was replaced with medium containing different concentrations of test substance (1:3 dilution steps) and plates were incubated at 37 °C and 5% CO₂. After 72 hours the medium was replaced by a solution of MTS cell viability dye (20 µL, Promega) in 150 µL of medium. Plates were incubated for 2 hours under culture conditions and the absorbance at 490 nm measured on a Spectra Max Paradigm multimode plate reader (Molecular Devices). Experiments were performed in triplicate and average cell viability calculated as measured background corrected absorbance divided by the absorbance of untreated control wells. IC₅₀ values were determined by fitting data to the four-parameter logistic equation, using a Prism 6 software (GraphPad Software) for data analysis.

1.4 Animal Studies
All animal experiments were conducted in accordance with Swiss animal welfare laws and regulations under the license number 27/2015 granted by the Veterinäramt des Kantons Zürich.
1.5 Implantation of Subcutaneous SKRC-52 Tumors
SKRC-52 cells were grown to 80% confluence and detached with Trypsin-EDTA 0.05% (Life Technologies). Cells were washed with Hank’s Balanced Salt Solution (HBSS, pH 7.4) once, counted and re-suspended in HBSS to a final concentration of $3.4 \times 10^7$ cells/mL. Aliquots of $5 \times 10^6$ cells (150 µL of a suspension) were injected subcutaneously in the right flank of female athymic BALB/c nu/nu mice (6-8 weeks of age, Janvier).

1.6 Therapy Experiments
SKRC-52 xenografted tumors were implanted into female BALB/c nu/nu mice (Janvier) as described above, and allowed to grow for two weeks to an average volume of 0.1 mL. Mice were randomly assigned into therapy groups of 4 animals and treatment started by injecting a solution of the targeted drugs or vehicle (PBS containing 1% of DMSO) intravenously (tail vein) at the dose of 250 nmol/Kg, following the schedule depicted by the arrows in the corresponding Figure. Compounds 2-5 were injected as solutions in sterile PBS containing 1% DMSO. Animals were weighed and tumor sizes were measured daily with an electronic caliper. The tumor volume was calculated according to the formula ($\text{long side} \times \text{short side} \times \text{short side} \times 0.5$). Animals were sacrificed when the termination criteria were reached. Prism 6 software (GraphPad Software) was used for data analysis (regular two-way ANOVA with the Bonferroni test).

1.7 IVIS Imaging
Female BALB/c nude mice bearing subcutaneous SKRC-52 tumors were injected intravenously with compound 6, containing the near infrared dye moiety IRDye680RD (LI-COR Biosciences) (250 nmol/Kg), dissolved in sterile PBS (100 µL). Mice were anesthetized with isoflurane and fluorescence images acquired on an IVIS Spectrum imaging system (Xenogen, exposure 1s, binning factor 8, excitation at 675 nm, emission filter at 720 nm, f number 2, field of view 13.1). Images were taken before
the injection and after 30 min, 1 and 3 hours. Food and water was given ad libitum during that period.

1.8 Immunofluorescence Studies
SKRC-52 tumors excised from the mice after therapy study were embedded in OCT medium (Thermo Scientific), and cryostat sections (10 µm) were cut. Slides were stored at -80 °C over-night, fixed with acetone and stained using the following antibodies: mouse anti-human CAIX GT12 (Thermo Scientific), to detect the antigen, and rat anti-mouse CD31 (BD Biosciences) to detect endothelial cells. Nuclear staining was performed with DAPI. Anti-mouse 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.
2. Chemical Procedures

2.1 General Chemical Procedures

Anhydrous solvents were purchased from Acros or Fluka. Peptide grade N,N-dimethylformamide (DMF) for solid phase synthesis was bought from ABCR. All other solvents were used as supplied by Fisher Chemicals, Merck or Sigma Aldrich in HPLC or analytical grade. H-Cys(Trt)-2-CT-polystyrene resin was purchased from RAPP Polymere. Maleimidocaproyl-ValCit-p-aminobenzylalcohol-MMAE (14d) and free MMAE were purchased from Levena Biopharma (No.9 Weidi Road, Qixia District, Nanjing, 210046, China). Maleimidoethyl-IRDye680RD was purchased from LI-COR Biosciences (4647 Superior Street Lincoln, Nebraska USA 68504-5000). All other reagents were purchased from Sigma Aldrich, Acros, ABCR or TCI and used as supplied. All reactions using anhydrous conditions were performed using oven-dried glassware under an atmosphere of argon. Silica for flash column chromatography (high-purity grade, pore size 60 Å) was purchased from Sigma Aldrich. Reactions were monitored by thin layer chromatography using Merck silica gel 60 F254 TLC glass plates. Concentration under reduced pressure was performed by rotary evaporation at 40 °C at the appropriate pressure. Yields refer to chromatographically purified and spectroscopically pure compounds, unless noted otherwise.

Proton (\(^1\)H) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV400 (400 MHz). Carbon (\(^{13}\)C) NMR spectra were recorded on a Bruker AV400 (100 MHz) spectrometer. Shifts are given in ppm using residual solvent as the internal standard. Coupling constants (\(J\)) are reported in Hz with the following abbreviations used to indicate splitting: \(s\) = singlet, \(d\) = doublet, \(t\) = triplet, \(q\) = quartet, \(m\) = multiplet.

High-Resolution Mass Spectrometry (HRMS) spectra and analytical Reversed-Phase Ultra Performance Liquid Chromatography (UPLC) were recorded on a Waters Xevo G2-XS QTOF coupled to a Waters Acquity UPLC H-Class System with PDA UV detector, using a ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 μm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min\(^{-1}\) with linear gradients of solvents A and B (A = Millipore water
with 0.1% formic acid [FA], B = MeCN with 0.1% formic acid [FA]). Preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) were performed on a Waters Alliance HT RP-HPLC with PDA UV detector, using a Synergi 4µm, Polar-RP 80Å 10 × 150 mm C18 column at a flow rate of 4 mL min⁻¹ with linear gradients of solvents A and B (A = Millipore water with 0.1% trifluoroacetic acid [TFA], B = MeCN with 0.1% trifluoroacetic acid [TFA]). Alternatively, a Waters Xterra 5 µm 150 Å 10 × 150 mm C18 column at a flow rate of 2.8 mL min⁻¹ was used with the same eluent phase.

Compound 1 [49] and Fmoc-Lys(Mtt)-PABOH [228] were prepared according to literature procedures and their analytical data were in agreement with those already published. The synthetic procedures for the preparation of the ValAla, ValLys, ValArg analogues, of the final SMDCs 2-5 and of the near-infrared dye derivative 6 are included in this chapter in the following sections, along with NMR/HPLC-MS data and the statistical analysis of the in vivo experiments.
Scheme VIII.1 Synthesis of Acetazolamide-MMAE conjugates 2-5. REAGENTS AND CONDITIONS: a) H-Val-OBu·HCl, EDC·HCl, DIPEA, CH₂Cl₂, 5 h, r.t., 82%; b) TFA:CH₂Cl₂ 2/1; 2h, 0 °C, 95%; c) PABOH, HATU, HOAt, DIPEA, DMF, 16 h, r.t., 65-85%; d) [1] 20% piperidine in DMF; [2] HATU, DIPEA, 0 °C to r.t., 16 h, 33-44%; e) 4-nitrophenyl chloroformate, pyridine, CH₂Cl₂, 2 h, r.t. (13a); 4-nitrophenyl carbonate, DIPEA, CH₂Cl₂, 48 h, r.t. (13b,c), 43-89%; f) MMAE-TFA; HOAt, DIPEA, DMF, 48 h, r.t., 41-46%; g) 1% TFA in CH₂Cl₂, 0 °C to r.t., 2 h, 55% (14b); TFA/TIS/water 94:3:3, 2.5 h, 0 °C, 52% (14c); h) 1, PBS/DMF 2:1, 3 h, r.t., 50-90%. 
2.2 Chemical Synthesis of Present Compounds

2.3.1 Synthesis of AAZ-ValAla-MMAE

MC-Val-OtBu – 8

![Chemical Structure](image)

Chemical Formula: C_{19}H_{30}N_{2}O_{5}
Molecular Weight: 366.46

Commercially available 6-maleimidohexanoic acid (7, 600 mg; 2.84 mmol; 1.0 eq) was dissolved in dry CH_{2}Cl_{2} (27 mL) under argon atmosphere and the solution was cooled to 0 °C. EDC·HCl (600 mg; 3.13 mmol; 1.1 eq), DIPEA (380 µL; 2.178 mmol; 2.3 eq) and H-Val-OtBu·HCl (654 mg; 3.13 mmol; 1.1 eq) were added subsequently. The reaction was stirred at room temperature for 5 hours. The mixture was diluted with AcOEt (100 mL), washed with KHSO_{4} (1M aqueous solution; 3 × 30 mL), NaHCO_{3} (saturated aqueous solution; 2 × 30 mL) and brine (1 × 20 mL). The organic phase was dried and concentrated, affording compound 8 as a pale, yellow oil (855 mg; 2.33 mmol; 82% yield).

{\^{1}}H NMR (400 MHz, DMSO-d6) δ 7.89 (d, J = 8.4 Hz, 1H), 7.00 (s, 2H), 4.04 (dd, J = 8.4, 6.0 Hz, 1H), 3.37 (t, J = 7.1 Hz, 2H), 2.18-2.07 (m, 2H), 1.98 (m, 1H), 1.53-1.42 (m, 4H), 1.39 (s, 9H), 1.26-1.12 (m, 2H), 0.86 (d, J = 4.3 Hz, 3H), 0.85 (d, J = 4.3 Hz, 3H) ppm; {\^{13}}C NMR (100 MHz, DMSO-d6) δ 172.3, 171.0, 170.8, 134.4, 80.3, 57.7, 37.0, 34.7, 29.9, 27.7, 27.6, 25.7, 24.9, 19.0, 18.1 ppm.
**MC-Val-OH – 9**

Chemical Formula: $C_{15}H_{22}N_2O_5$
Molecular Weight: 310.35

*tert*-butyl ester 8 (855 mg; 2.33 mmol; 1.0 eq) was dissolved in dry CH$_2$Cl$_2$ (8 mL) under argon atmosphere. TFA (4 mL; 51.26 mmol; 22.0 eq) was added and the mixture was stirred at room temperature for 2 hours. The solution was concentrated and precipitated with hexane, affording carboxylic acid 9 as a yellow solid (685 mg; 2.21 mmol; 95% yield).

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.90 (d, $J = 8.6$ Hz, 1H), 7.01 (s, 2H), 4.13 (dd, $J = 8.6$, 5.9 Hz, 1H), 3.38 (t, $J = 7.1$ Hz, 2H), 2.23-2.07 (m, 2H), 2.02 (m, 1H), 1.55-1.42 (m, 4H), 1.25-1.14 (m, 2H), 0.87 (d, $J = 6.8$ Hz, 3H), 0.86 (d, $J = 6.8$ Hz, 3H) ppm; $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 173.7, 172.8, 171.5, 134.9, 57.5, 37.5, 35.2, 30.2, 28.2, 26.2, 25.3, 19.6, 18.5 ppm.
**Fmoc-Ala-PABOH – 11a**

![Chemical Structure](image)

Chemical Formula: C$_{25}$H$_{24}$N$_{2}$O$_{4}$

Molecular Weight: 416.48

Fmoc-Ala-OH (10a; 312 mg; 1.00 mmol; 1.0 eq) was dissolved in dry DMF (4 mL) under argon atmosphere and the solution was cooled to 0 °C. HATU (420 mg; 1.11 mmol; 1.1 eq), HOAt (151 mg; 1.11 mmol; 1.1 eq) and DIPEA (520 µL; 2.98 mmol; 3.0 eq) were added subsequently. The mixture was stirred at the same temperature for 15 minutes. 4-aminobenzyl alcohol was added as a solution in dry DMF (2 mL). The solution was allowed to reach room temperature and stirred over-night. The mixture was diluted with AcOEt (100 mL) and washed with KHSO$_4$ (1M aqueous solution; 3 × 20 mL), NaHCO$_3$ (saturated aqueous solution; 3 × 20 mL) and brine (3 × 20 mL). The organic phase was dried and concentrated under vacuum. The crude mixture was purified via flash column (gradient from 99:1 to 95:5 CH$_2$Cl$_2$/MeOH) to afford compound 11a (274 mg; 0.66 mmol; 66% yield).

R$_f$ = 0.39 (CH$_2$Cl$_2$/MeOH, 95:5); $^1$H NMR (400 MHz, DMSO-$_d$6) δ 9.96 (s, 1H), 7.89 (d, $J$ = 7.6 Hz, 2H), 7.79-7.72 (m, 2H), 7.68 (d, $J$ = 7.4 Hz, 1H), 7.58 (d, $J$ = 8.2 Hz, 2H), 7.46-7.39 (m, 2H), 7.38-7.32 (m, 2H), 7.26 (d, $J$ = 8.2 Hz, 2H), 5.12 (t, $J$ = 5.6 Hz, 1H), 4.45 (d, $J$ = 5.6 Hz, 2H), 4.32-4.28 (m, 2H), 4.28-4.16 (m, 2H), 1.33 (d, $J$ = 7.2 Hz, 3H) ppm; $^{13}$C NMR (100 MHz, DMSO-$_d$6) δ 171.9, 156.2, 144.4, 144.3, 141.2, 138.1, 137.8, 128.1, 127.5, 127.3, 125.8, 125.8, 120.6, 120.5, 119.4, 66.1, 63.1, 51.2, 47.1, 18.6 ppm.
MC-ValAla-PABOH – 12a

Chemical Formula: C$_{25}$H$_{34}$N$_4$O$_6$
Molecular Weight: 486.57

Compound 11a (274 mg; 0.66 mmol) was dissolved in dry DMF (30 mL) under argon atmosphere and cooled to 0 °C. Piperidine (325 µL; 3.29 mmol) was added and the mixture was stirred at room temperature for 1 hour. The solution was concentrated under high vacuum, dissolved in AcOEt (100 mL), washed with NaHCO$_3$ (saturated aqueous solution; 2 × 20 mL) and brine (2 × 20 mL). The organic phase was dried and concentrated, The crude material was dissolved in AcOEt:Hexane (1:1; 30 mL) and purified over a pad of silica (gradient from 1:1 AcOEt/Hexane to 9:1 CH$_2$Cl$_2$/MeOH with 0.5% TEA), to afford NH$_2$-Ala-PABOH as a brown oil (60 mg; 0.31 mmol).

Carboxylic acid 9 (105 mg; 0.34 mmol; 1.1 eq) was dissolved in dry DMF (4 mL) under argon atmosphere and the solution was cooled to 0 °C. HATU (129 mg; 0.34 mmol; 1.1 eq), NH$_2$-Ala-PABOH (60 mg; 0.31 mmol; 1.0 eq) and DIPEA (80 µL; 0.46 mmol; 1.5 eq) were added subsequently. The reaction was allowed to slowly reach room temperature and stirred overnight. DMF was removed under vacuum. The crude was dissolved in AcOEt (80 mL) and washed with KHSO$_4$ (1 M aqueous solution; 2 × 20 mL) and brine (1 × 20 mL). The organic phase was dried and concentrated under vacuum. The crude was purified via flash column (eluent: 93:7 CH$_2$Cl$_2$/MeOH) to afford compound 12a (55 mg; 0.11 mmol; 33% yield).
$R_f = 0.22$ (CH$_2$Cl$_2$/MeOH, 92:8); $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 9.71 (s, NH residue), 8.28 (d, $J = 6.8$ Hz, NH residue), 7.96 (d, $J = 7.9$ Hz, NH residue), 7.55 (d, $J = 8.5$ Hz, 2H), 7.29 (d, $J = 8.6$ Hz, 2H), 6.78 (s, 2H), 4.55 (s, 2H), 4.48 (m, 1H), 4.18 (m, 1H), 3.47 (t, $J = 7.1$ Hz, 2H), 2.27 (t, $J = 7.4$ Hz, 2H), 2.08 (m, 1H), 1.69-1.52 (m, 4H), 1.44 (d, $J = 7.1$ Hz, 3H), 0.98 (d, $J = 6.8$ Hz, 3H), 0.96 (d, $J = 6.8$ Hz, 3H) ppm; $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 174.9, 172.3, 171.5, 171.1, 137.4, 137.2, 133.9, 127.2, 119.8, 63.4, 59.0, 49.6, 37.0, 35.1, 30.4, 27.9, 26.0, 25.0, 18.3, 17.4, 16.7 ppm.
MC-ValAla-PABC-PNP – 13a

A solution of 4-Nitrophenyl chloroformate (47 mg; 0.27 mmol; 2.2 eq) in dry CH$_2$Cl$_2$ (300 µL) was added under argon atmosphere to a suspension of compound 12a (59 mg; 0.12 mmol; 1.0 eq) in dry CH$_2$Cl$_2$ (2 mL) and pyridine (15 µL; 0.185 mmol; 1.5 eq). DMF (250 µL) was added to the reaction mixture to help dissolution. The reaction completion was monitored by TLC (eluent 9:1 CH$_2$Cl$_2$/MeOH) and the solvent was removed after 2 hours. The crude mixture was purified over a pad of silica (AcOEt) to give compound 13a as a white solid (67 mg; 0.10 mmol; yield 86%).

$R_f = 0.58$ (AcOEt); HRMS: (m/z) [M+H]$^+$ C$_{32}$H$_{38}$N$_5$O$_{10}$, 652.2613, found 652.2667; [M+Na]$^+$ C$_{32}$H$_{37}$N$_5$NaO$_{10}$, 674.2433, found 674.2511.
Monomethyl auristatin E (MMAE-TFA; 10 mg; 0.012 mmol; 1.1 eq) was dissolved in dry DMF (200 µL) under nitrogen atmosphere. Compound 13a (7 mg; 0.011 mmol; 1.0 eq), HOAt (0.7 mg; 0.006 mmol; 0.5 eq) and DIPEA (10 µL; 0.055 mmol; 5.0 eq) were added subsequently. The mixture was stirred at room temperature for 48 hours and concentrated under vacuum. The crude was diluted with a H₂O/MeOH 1:1 mixture (200 µL), and purified over RP-HPLC (Waters Xterra 5 µm 150 Å 10 ×150 mm C18 column, 5% MeCN in 0.1% aq. TFA to 80% over 30 min). product-containing fractions were identified by mass spectrometry and lyophilized overnight to obtain compound 14a as a white solid (5.5 mg, 4.5 µmol, 41% yield).

HRMS: \(m/z\) \([\text{M+H}]^+\) \(\text{C}_{65}\text{H}_{99}\text{N}_9\text{O}_{14}\), 1230.7384, found 1230.7805; \([\text{M+Na}]^+\) \(\text{C}_{66}\text{H}_{100}\text{N}_9\text{NaO}_{14}\), 1252.7209, found 1252.7644.
Figure IX.1 Analytical UPLC trace of compound 14a on a BEH C18 Column, 130 Å, 1.7 μm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
AAZ-ValAla-MMAE – 2

Chemical Formula: C_{95}H_{145}N_{23}O_{27}S_3
Molecular Weight: 2137.52

Compound 1 (6.0 mg; 6.6 µmol; 4.0 eq) was dissolved in degassed PBS (pH 7.4; 600 µl). MC-ValAla-MMAE (14a; 2.2 mg, 1.6 µmol; 1.0 eq) was added as a DMF solution (350 µl) and the mixture was stirred at room temperature. Product formation was monitored by UPLC-MS. After 3 hours, the solvents were removed under vacuum. The crude was diluted in a 1:1 H_{2}O/MeCN mixture (1 mL) and purified by RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). Pure fractions were collected and lyophilized overnight to achieve compound 2 as a white solid (2.3 mg; 1.1 µmol; 66% yield).

HRMS: (m/z) [M+2H]^{2+} C_{95}H_{147}N_{23}O_{27}S_3, 1068.9994, found 1069.0012.
**Figure IX.2** Analytical UPLC trace of AAZ-ValAla-MMAE (compound 2) on a BEH C18 Column, 130 Å, 1.7 μm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
2.3.2 Synthesis of AAZ-ValLys-MMAE

MC-ValLys(Mtt)-PABOH – 12b

Chemical Formula: C₄₈H₅₇N₅O₆
Molecular Weight: 800.01

Compound 11a (213 mg; 0.29 mmol) was dissolved in dry DMF (14 mL) under argon atmosphere and cooled to 0 °C. Piperidine (143 µL; 1.45 mmol) was added and the mixture was stirred at room temperature for 1 hour. The solution was concentrated under high vacuum and the crude was dissolved in AcOEt (100 mL). The solution was washed with NaHCO₃ (saturated aqueous solution; 2 × 20 mL) and brine (2 × 20 mL). The organic phase was dried and concentrated. The mixture was dissolved in a 1:1 AcOEt:Hexane mixture (30 mL) and filtered over a pad of silica (gradient from 1:1 AcOEt:Hexane to 9:1 CH₂Cl₂/MeOH + 0.5% TEA) to afford NH₂-Lys(Mtt)-PABOH as a brown oil (125 mg; 0.25 mmol).

Compound 9 (13 mg; 0.043 mmol; 1.1 eq) was dissolved in dry DMF (700 µL) under argon atmosphere and the solution was cooled to 0 °C. HATU (16.4 mg; 0.043 mmol; 1.1 eq), and DIPEA (10 µL; 0.059 mmol; 1.5 eq) were added subsequently and allowed to react for 15 minutes at 0 °C. NH₂-Lys(Mtt)-PABOH (20 mg; 0.039 mmol; 1.0 eq) was dissolved in dry DMF (100 µL) and added to the stirring solution. The reaction was allowed to reach room temperature and stirred for 6 hours. The reaction mixture was diluted with AcOEt (40 mL) and washed with KHSO₄ (1 M
aqueous solution; 2 × 5 mL). The organic phase was dried and concentrated under vacuum. The crude material was purified by flash column (eluent: 96:4 CH₂Cl₂/MeOH) to afford compound 12b (28 mg; 0.0.035 mmol; 90% yield).

Rₚ = 0.37 (AcOEt/Hexane 9:1); ¹H NMR (400 MHz, DMSO-d6) δ 9.86 (s, 1H), 7.97 (d, J = 7.7 Hz, 2H), 7.79 (d, J = 8.6 Hz, 2H), 7.52 (d, J = 8.6 Hz, 2H), 7.40-7.33 (m, 4H), 7.28-7.24 (m, 4H), 7.24-7.20 (m, 4H), 7.17-7.12 (m, 2H), 7.05 (d, J = 8.0 Hz, 2H), 6.99 (s, 2H), 5.08 (t, J = 5.6 Hz, 1H), 4.42 (d, J = 5.6 Hz, 2H), 4.38-4.30 (m, 1H), 4.15 (dd, J = 8.6, 6.9 Hz, 1H), 3.35 (t, J = 7.2 Hz, 2H), 2.41 (t, J = 8.0 Hz, 1H), 2.23 (s, 3H), 2.21-2.02 (m, 2H), 2.00-1.86 (m, 3H), 1.66 (m, 1H), 1.56 (m, 1H), 1.52-1.39 (m, 5H), 1.37-1.22 (m, 1H), 1.38-1.12 (m, 2H), 0.83 (d, J = 6.8 Hz, 3H), 0.80 (d, J = 6.8 Hz, 3H) ppm; ¹³C NMR (101 MHz, DMSO-d6) δ 172.2, 171.1, 171.0, 170.4, 146.4, 146.4, 143.2, 137.5, 137.4, 134.9, 134.4, 128.3, 128.2, 127.6, 126.9, 125.9, 118.9, 70.1, 62.6, 57.7, 53.4, 43.2, 37.0, 34.9, 32.0, 30.3, 29.8, 27.8, 25.8, 24.9, 23.5, 20.5, 19.2, 18.2 ppm.
MC-ValLys(Mtt)-PABC-PNP – 13b

Chemical Formula: C_{55}H_{60}N_{6}O_{10}
Molecular Weight: 965.12

Compound 12b (28 mg; 0.035 mmol; 1.0 eq) was suspended in 1.8 mL of dry CH\textsubscript{2}Cl\textsubscript{2} under argon atmosphere. DIPEA (20 µL; 0.115 mmol; 3.3 eq) and a solution of bis(4-nitrophenyl) carbonate (33 mg; 0.108 mmol; 3.1 eq) in dry CH\textsubscript{2}Cl\textsubscript{2} (500 µL) were added to the reaction mixture. After 2 days TLC (silica, 9:1 AcOEt/Hexane) indicated completion. Solvents were removed and the crude purified via flash column (silica, 7:3 AcOEt:Hexane) to give compound 13b as a white solid (30 mg; 0.031; yield 89%).

R\textsubscript{f} = 0.28 (AcOEt/Hexane 6:4)

HRMS: (m/z) [M+H]\textsuperscript{+} C_{55}H_{61}N_{6}O_{19}, 965.4444, found 965.4456; [M+Na]\textsuperscript{+} C_{55}H_{60}N_{6}NaO_{19}, 987.4263, found 987.4280; [M+H-Mtt]\textsuperscript{+} C_{35}H_{44}N_{6}O_{10}, 709.3192, found 709.3217.
Monomethyl auristatin E (MMAE·TFA; 18 mg; 0.021 mmol; 1.0 eq) was dissolved in dry DMF (400 µL) under nitrogen atmosphere. Carbonate 13b (30 mg; 0.031 mmol; 1.5 eq), HOAt (2 mg; 0.015 mmol; 0.5 eq) and DIPEA (27 µL; 0.155 mmol; 7.3 eq) were added subsequently. The mixture was stirred at room temperature for 6 days and concentrated under vacuum. The crude was diluted with water (100 µL) and MeOH (100 µL), and purified over RP-HPLC (Waters Xterra 5 µm 150 Å 10 × 150 mm C18 column, 5% MeCN in 0.1% aq. TFA to 80% over 30 min) and product-containing fractions identified by mass spectrometry and lyophilized overnight to obtain compound 14bP as a white solid (15 mg, 9.7 µmol, 46% yield).

HRMS: (m/z) [M+H]+ C_{88}H_{123}N_{10}O_{14}, 1543.9215, found 1543.9899; [M+Na]+ C_{88}H_{122}N_{10}NaO_{14}, 1565.9034, found 1565.9729; [M+H-Mtt]+ C_{68}H_{107}N_{10}O_{14}, 1287.7963, found 1287.8427.
MC-ValLys-MMAE – 14b

Chemical Formula: $C_{68}H_{106}N_{10}O_{14}$
Molecular Weight: 1287.65

Compound 14bP (10 mg; 6.5 µmol; 1.0 eq) was dissolved in CH$_2$Cl$_2$ (3 mL) and cooled to 0 °C. TFA (30 µL) was added to the solution. The mixture was allowed to reach the room temperature and stirred for 2 hours. The solvent was evaporated and then for two times CH$_2$Cl$_2$ was added to the residue followed by evaporation under vacuum, to afford the amine TFA salt. The latter was diluted with a H$_2$O/MeOH 1:1 mixture (200 µL), and purified over RP-HPLC (Waters Xterra 5 µm 150 Å 10 × 150 mm C18 column, 5% MeCN in 0.1% aq. TFA to 80% over 30 min). Pure fractions were collected and lyophilized overnight to obtain compound 14b as a white solid (5 mg, 3.6 µmol, 55% yield).

HRMS: ($m/z)$ [M+H]$^+$ $C_{68}H_{107}N_{10}O_{14}$, 1287.7963, found 1287.8435; [M+Na]$^+$ $C_{68}H_{106}N_{10}NaO_{14}$, 1309.7782, found 1309.8271; [M+2H]$^{2+}$ $C_{68}H_{108}N_{10}O_{14}$, 644.4018, found 644.4070.
Figure IX.3 Analytical UPLC trace of compound 14b on a BEH C18 Column, 130 Å, 1.7 μm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
AAZ-ValLys-MMAE – 3

Chemical Formula: C_{68}H_{106}N_{10}O_{14}
Molecular Weight: 1287.65

Compound 1 (5.8 mg; 6.4 µmol; 4.0 eq) was dissolved in degassed PBS (pH 7.4; 600 µl). Compound 14b (2.0 mg, 1.6 µmol; 1.0 eq) was added as a DMF solution (350 µl) and the mixture was stirred at room temperature. After 3 hours, UPLC-MS indicated completion. The solvents were removed under vacuum and the crude was diluted in a 1:1 H_{2}O/MeCN mixture (1 mL). The mixture was purified by RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). Pure fractions were collected and lyophilized overnight to achieve 3 as a white solid (2.0 mg; 0.9 µmol; 59% yield).

HRMS: (m/z) [M+2H]^{2+} C_{98}H_{154}N_{24}O_{17}S_{3}, 1097.5283, found 1097.5287; [M+3H]^{3+} C_{98}H_{155}N_{24}O_{17}S_{3}, 732.0213, found 732.0220.
Figure IX.4 Analytical UPLC trace of AAZ-Val-Lys-MMAE (compound 3) on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
2.3.3 Synthesis of AAZ-ValArg-MMAE

*Fmoc-Arg(Pbf)-PABOH – 11c*

![Chemical Structure](image)

Chemical Formula: C_{41}H_{47}N_{5}O_{7}S
Molecular Weight: 753.20

Fmoc-Arg(Pbf)-OH (10c; 300 mg; 0.46 mmol; 1.0 eq) was dissolved in dry DMF (8 mL) under argon atmosphere and the solution was cooled to 0 °C. HATU (193 mg; 0.51 mmol; 1.1 eq), HOAt (69 mg; 0.51 mmol; 1.1 eq) and DIPEA (240 µL; 1.40 mmol; 3.0 eq) were added subsequently, and the mixture was stirred at 0 °C for 15 minutes. 4-aminobenzyl alcohol was dissolved in dry DMF (2 mL) and added to the stirring mixture, which was then allowed to reach room temperature overnight. AcOEt (100 mL) was added and the solution was washed with KHSO_{4} (1 M aqueous solution; 3 × 20 mL), NaHCO_{3} (saturated aqueous solution; 3 × 20 mL) and brine (3 × 20 mL). The organic phase was dried and concentrated under vacuum. The crude material was purified by flash column (eluent 95:5 CH_{2}Cl_{2}/MeOH), to afford compound 11c (285 mg; 0.38 mmol; 82% yield).

R_{f} = 0.10 (CH_{2}Cl_{2}/MeOH, 98:2); \textsuperscript{1}H NMR (400 MHz, DMSO-d_{6}) \delta 9.99 (s, 1H), 7.89 (d, J = 7.5 Hz, 2H), 7.74 (t, J = 6.4 Hz, 2H), 7.66 (d, J = 8.0 Hz, 1H), 7.55 (d, J = 8.3 Hz, 2H), 7.41 (dd, J_{1} = J_{2} = 7.5 Hz, 2H), 7.36-7.29 (m, 2H), 7.24 (d, J = 8.3 Hz, 2H), 6.70 (bs, 1H), 6.38 (bs, 1H), 5.10 (bs, 1H), 4.44 (s, 2H), 4.32-4.25 (m, 2H), 4.22 (m, 1H), 4.14 (m, 1H), 3.33 (bs,
1H), 3.11-3.02 (m, 2H), 2.91 (s, 2H), 2.47 (s, 3H), 2.41 (s, 3H), 1.98 (s, 3H), 1.69 (m, 1H), 1.60 (m, 1H), 1.48 (m, 1H), 1.38 (s, 6H), 1.23 (m, 1H) ppm; $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 170.7, 157.4, 156.0, 143.8, 143.7, 140.7, 137.5, 137.3, 131.4, 127.6, 127.0, 126.9, 125.3, 124.3, 120.1, 118.9, 116.2, 86.3, 65.7, 62.6, 55.0, 46.7, 42.4, 39.6 (overlapped with solvent signal), 29.4, 28.3, 25.8, 18.9, 17.6, 12.3 ppm.
MC-ValArg(Pbf)-PABOH – 12c

Chemical Formula: \(C_{41}H_{57}N_7O_9S\)
Molecular Weight: 824.01

Compound 11c (285 mg; 0.38 mmol) was dissolved in dry DMF (15 mL) under argon atmosphere and cooled to 0 °C. Piperidine (186 µL; 1.89 mmol) was added and the mixture was stirred at room temperature for 1 hour. The solution was concentrated under high vacuum and the crude material was dissolved in AcOEt (100 mL). The solution was washed with \(\text{NaHCO}_3\) (saturated aqueous solution; 2 × 20 mL) and brine (2 × 20 mL). The organic phase was dried and concentrated, and the crude material was dissolved in AcOEt:Hexane (1:1; 30 mL) and filtered over a pad of silica (gradient from 1:1 AcOEt/Hexane to 9:1 \(\text{CH}_2\text{Cl}_2/\text{MeOH}\) with 0.5% TEA) to afford NH2-Arg(Pbf)-PABOH (100 mg; 0.19 mmol).

Compound 9 (63 mg; 0.21 mmol; 1.1 eq) was dissolved in dry DMF (4 mL) under argon atmosphere and the solution was cooled to 0 °C. HATU (78 mg; 0.21 mmol; 1.1 eq) and NH2-Arg(Pbf)-PABOH (100 mg; 0.19 mmol; 1.0 eq) were added subsequently. The reaction was allowed to reach room temperature and stirred overnight. DMF was removed under vacuum. The crude was dissolved in AcOEt (70 mL) and washed with \(\text{KHSO}_4\) (1 M aqueous solution; 2 × 10 mL). The organic phase was dried and concentrated. The crude material was purified via flash column (94:6 \(\text{CH}_2\text{Cl}_2/\text{MeOH}\)) to afford compound 12c (68 mg; 0.08 mmol; 44% yield).
$R_f = 0.38$ (CH$_2$Cl$_2$/MeOH 9:1); $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.55 (d, $J = 8.5$ Hz, 2H), 7.30 (d, $J = 8.5$ Hz, 2H), 6.78 (s, 2H), 4.56 (s, 2H), 4.50 (dd, $J = 9.0$, 5.2 Hz, 1H), 4.12 (d, $J = 7.6$ Hz, 1H), 3.46 (t, $J = 7.1$ Hz, 2H), 2.97 (s, 2H), 2.56 (s, 3H), 2.49 (s, 3H), 2.26 (t, $J = 7.4$ Hz, 2H), 2.05 (s, 3H), 2.02 (m, 1H), 1.88 (m, 1H), 1.74 (m, 1H), 1.66-1.52 (m, 4H), 1.44 (s, 6H), 1.37-1.24 (m, 6H), 0.96 (d, $J = 6.8$ Hz, 6H) ppm; $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 159.9, 139.4, 138.8, 135.3, 133.5, 128.6, 126.0, 121.3, 87.7, 64.8, 60.7, 54.8, 44.0, 38.4, 36.5, 31.6, 30.5, 29.3, 28.7, 27.4, 26.4, 19.8, 19.6, 19.0, 18.4, 12.5 ppm.
MC-ValArg(Pbf)-PABC-PNP – 13c

Chemical Formula: C\textsubscript{48}H\textsubscript{60}N\textsubscript{8}O\textsubscript{13}S
Molecular Weight: 989.11

Compound 12c (59 mg; 0.07 mmol; 1.0 eq) was suspended in 2.5 mL of dry CH\textsubscript{2}Cl\textsubscript{2} under argon atmosphere and DIPEA (37 µL; 0.21 mmol; 3.0 eq) was added. A solution of bis(4-nitrophenyl) carbonate (95 mg; 0.31 mmol; 4.4 eq) in dry CH\textsubscript{2}Cl\textsubscript{2} (500 µL) was added to the reaction mixture, which was stirred overnight at room temperature. After 48 hours, TLC (9:1 CH\textsubscript{2}Cl\textsubscript{2}/MeOH) indicated completion. Solvents were removed by evaporation under vacuum to give a yellow crude material, which was purified over a pad of silica (gradient from 1:1 AcOEt/Hexane to 1:1 AcOEt/Acetone) to give compound 13c as a white solid (30 mg; 0.03; 43% yield).

R\textsubscript{f} = 0.52 (CH\textsubscript{2}Cl\textsubscript{2}/MeOH 9:1).

HRMS: (m/z) [M+H]\textsuperscript{+} C\textsubscript{48}H\textsubscript{61}N\textsubscript{8}O\textsubscript{13}S, 989.4073, found 989.3915; [M+Na]\textsuperscript{+} C\textsubscript{48}H\textsubscript{60}N\textsubscript{8}NaO\textsubscript{13}S, 1011.3893, found 1011.4202.
Monomethyl auristatin E (MMAE·TFA; 20 mg; 0.024 mmol; 1.1 eq) was dissolved in dry DMF (400 µL) under nitrogen atmosphere. Compound 13c (22 mg; 0.022 mmol; 1.0 eq), HOAt (0.15 mg; 0.011 mmol; 0.5 eq) and DIPEA (19 µL; 0.11 mmol; 5.0 eq) were added subsequently. The mixture was stirred at room temperature for 48 hours and concentrated under vacuum. The crude was diluted with water (100 µL) and MeOH (100 µL), and purified over RP-HPLC (Waters Xterra 5 µm 150 Å 10 × 150 mm C18 column, 5% MeCN in 0.1% aq. TFA to 80% over 30 min) and product-containing fractions identified by mass spectrometry and lyophilized overnight to obtain compound 14cP as a white solid (15 mg, 9.5 µmol, 43% yield).

HRMS: (m/z) [M+H]^+ C_{81}H_{123}N_{12}O_{17}S, 1567.8844, found 1567.9597; [M+Na]^+ C_{81}H_{122}N_{12}NaO_{17}S, 1589.8664, found 1589.9468.
MC-ValArg-MMAE – 14c

Chemical Formula: C_{68}H_{106}N_{12}O_{14}
Molecular Weight: 1315.67

Compound 14cP (15 mg, 9.5 µmol; 1.0 eq) was flushed with argon and cooled to 0 °C. A mixture of TFA/TIS/water (94:3:3; 1 mL) was added and the reaction was stirred for 2.5 hours at 0 °C. The solvent was evaporated and then for two times CH$_2$Cl$_2$ was added to the residue followed by evaporation under vacuum, to afford the relative TFA salt. The latter was diluted with water (100 µL) and MeOH (100 µL), and purified over RP-HPLC (Waters Xterra 5 µm 150 Å 10 × 150 mm C18 column, 5% MeCN in 0.1% aq. TFA to 80% over 30 min). Pure fractions were identified by mass spectrometry and lyophilized overnight to obtain compound 14c as a white solid (6.4 mg, 4.9 µmol, 52% yield).

HRMS: ($m/z$) [M+H]$^+$ C$_{68}$H$_{107}$N$_{12}$O$_{14}$, 1315.8024, found 1316.8059; [M+Na]$^+$ C$_{68}$H$_{107}$N$_{12}$NaO$_{14}$, 1336.7765, found 1337.7842.
Figure IX.5 Analytical UPLC trace of compound 14c on a BEH C18 Column, 130 Å, 1.7 μm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
AAZ-ValArg-MMAE – 4

Chemical Formula: C\textsubscript{98}H\textsubscript{152}N\textsubscript{26}O\textsubscript{27}S

Molecular Weight: 2222.63

Compound 1 (4.0 mg; 4.4 µmol; 4.0 eq) was dissolved in degassed PBS (pH 7.4; 600 µl). Compound 14c (1.5 mg, 1.1 µmol; 1.0 eq) was added as a DMF solution (350 µl) and the mixture was stirred at room temperature. After 3 hours, UPLC-MS indicated completion. The solvents were removed under vacuum and the crude was diluted in a 1:1 H\textsubscript{2}O/MeCN mixture. The crude was purified by RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). Pure fractions were collected and lyophilized overnight to afford compound 4 as a white solid (2.4 mg; 1.08 µmol; 94% yield).

HRMS: (m/z) [M+2H]\textsuperscript{2+} C\textsubscript{98}H\textsubscript{154}N\textsubscript{26}O\textsubscript{27}S\textsubscript{3}, 1111.5314, found 1111.5288; [M+3H]\textsuperscript{3+} C\textsubscript{98}H\textsubscript{155}N\textsubscript{26}O\textsubscript{27}S\textsubscript{3}, 741.3567, found 741.3563.
**Figure IX.6** Analytical UPLC trace of AAZ-ValArg-MMAE (compound 4) on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
2.3.4 Synthesis of AAZ-ValCit-MMAE

AAZ-ValCit-MMAE – 5

Chemical Formula: C$_{98}$H$_{151}$N$_{25}$O$_{28}$S$_3$
Molecular Weight: 2223.61

Compound 5 was synthesized following the procedure previously described [132]. Briefly, to a solution of compound 1 (4.5 mg, 5 µmol) in degased PBS (pH 7.4; 1 mL) was added the commercially available MC-ValCit-PABCA-MMAE (14d; 6.5 mg, 4.9 µmol) dissolved in DMF (1 mL). The mixture was stirred at room temperature for 5 min and the solvents were removed under vacuum. The conjugate was purified over RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min) and product containing fractions identified by mass spectrometry and lyophilized overnight to obtain a white solid (7.5 mg, 3.4 µmol, 68%).

HRMS: (m/z) [M+2H]$^{2+}$ C$_{98}$H$_{153}$N$_{25}$O$_{28}$S$_3$, 1112.0234, found 1112.0237.
Figure IX.7 Analytical UPLC trace of AAZ-ValCit-MMAE (compound 5) on a BEH C18 Column, 130 Å, 1.7 μm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
2.3.5 Synthesis of AAZ-IRdye680RD

AAZ-IRdye680RD – 6

Chemical Formula: C_{75}H_{97}ClN_{19}O_{25}S_{6}^+

Molecular Weight: 1892.52

Compound 6 was synthesized following the procedure previously described [132].
2.3 $^1$H and $^{13}$C NMR spectra

MC-Val-OtBu – (1)
MC-Val-OH – (2)
Fmoc-Ala-PABOH – (3)

[Diagram of NMR spectrum]

[Diagram of 1H NMR spectrum]

[Diagram of 13C NMR spectrum]

179
MC-Val-Lys(Mtt)-PABOH – (12)
3. Statistical analysis of therapy experiment

Differences in tumor volume between therapeutic groups were compared using the two-way ANOVA analysis with Bonferroni post-test of Graphpad Prism 6 (La Jolla, CA, USA).

Tumor Size (mm$^3$)

**Compound 2** (AAZ-ValAla-MMAE) vs. Vehicle:
- day 10 \( p < 0.05 \)
- day 11 \( p < 0.05 \)
- day 12 \( p < 0.001 \)
- from day 13 \( p < 0.0001 \)

**Compound 3** (AAZ-ValLys-MMAE) vs. Vehicle:
- day 12 \( p < 0.01 \)
- day 13 \( p < 0.001 \)
- from day 14 \( p < 0.0001 \)

**Compound 4** (AAZ-ValArg-MMAE) vs. Vehicle:
- day 14 \( p < 0.001 \)
- from day 15 \( p < 0.0001 \)

**Compound 5** (AAZ-ValCit-MMAE) vs. Vehicle:
- day 11 \( p < 0.05 \)
- day 12 \( p < 0.01 \)
- day 13 \( p < 0.001 \)
- from day 14 \( p < 0.0001 \)

**Compound 2** (AAZ-ValAla-MMAE) vs compound 3 (AAZ-ValLys-MMAE):
- day 23 \( p < 0.01 \)
- day 24 \( p < 0.001 \)
- from day 25 \( p < 0.0001 \)

**Compound 2** (AAZ-ValAla-MMAE) vs compound 4 (AAZ-ValArg-MMAE):
day 21 \( p < 0.01 \)
day 22 \( p < 0.01 \)
from day 23 \( p < 0.0001 \)

Compound 2 (AAZ-ValAla-MMAE) vs. compound 5 (AAZ-ValCit-MMAE):
day 32 \( p < 0.001 \)
day 33 \( p < 0.05 \)
day 34 non-significant differences
day 35 non-significant differences
from day 36 \( p < 0.01 \)

Compound 3 (AAZ-ValLys-MMAE) vs. compound 4 (AAZ-ValArg-MMAE):
non-significant differences

Compound 3 (AAZ-ValLys-MMAE) vs. compound 5 (AAZ-ValCit-MMAE):
day 21 \( p < 0.01 \)
day 22 \( p < 0.01 \)
day 23 \( p < 0.01 \)
day 24 \( p < 0.05 \)
day 25 \( p < 0.05 \)
day 26 \( p < 0.001 \)
day 27 \( p < 0.001 \)
from day 28 \( p < 0.0001 \)

Compound 4 (AAZ-ValArg-MMAE) vs. compound 5 (AAZ-ValCit-MMAE):
day 21 \( p < 0.05 \)
day 22 \( p < 0.05 \)
day 23 \( p < 0.001 \)
day 24 \( p < 0.01 \)
from day 25 \( p < 0.0001 \)
Body Weight Change (%)

Compound 2 (AAZ-ValAla-MMAE) vs. Vehicle: 
non-significant differences

Compound 3 (AAZ-ValLys-MMAE) vs. Vehicle: 
non-significant differences

Compound 4 (AAZ-ValArg-MMAE) vs. Vehicle: 
non-significant differences

Compound 5 (AAZ-ValCit-MMAE) vs. Vehicle:

day 17  p < 0.05
day 18  p < 0.01
day 19  p < 0.05
day 20  P < 0.05
from day 21  non-significant differences

Compound 2 (AAZ-ValAla-MMAE) vs. compound 3 (AAZ-ValLys-MMAE):
non-significant differences

Compound 2 (AAZ-ValAla-MMAE) vs. compound 4 (AAZ-ValArg-MMAE):
non-significant differences

Compound 2 (AAZ-ValAla-MMAE) vs. compound 5 (AAZ-ValCit-MMAE):
non-significant differences

Compound 3 (AAZ-ValLys-MMAE) vs. compound 4 (AAZ-ValArg-MMAE):
non-significant differences

Compound 3 (AAZ-ValLys-MMAE) vs. compound 5 (AAZ-ValCit-MMAE):
day 17  p < 0.05
day 18  p < 0.05
from day 19  non-significant differences

Compound 4 (AAZ-ValArg-MMAE) vs. compound 5 (AAZ-ValCit-MMAE):
<table>
<thead>
<tr>
<th>Day</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 19</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>day 20</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>from day 21</td>
<td>non-significant differences</td>
</tr>
</tbody>
</table>
1. Biological Procedures

1.1 Cell Cultures

The human renal cell carcinoma cell line SKRC-52 was kindly provided by Professor E. Oosterwijk (Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands). Upon thawing, cells were kept in culture for no longer than 10 passages. SKRC-52 were maintained in RPMI medium (Invitrogen) supplemented with fetal calf serum (10%, FCS, Invitrogen) and Antibiotic-Antimycotic (1%, AA, Invitrogen) at 37 °C and 5% CO₂. For passaging, cells were detached using Trypsin-EDTA 0.05% (Invitrogen) when reaching 90% confluence and re-seeded at a dilution of 1:6.

The murine colorectal carcinoma cell line CT26.wt (ATCC) was maintained in RPMI medium (Invitrogen) supplemented with fetal calf serum (10%, FCS, Invitrogen) and Antibiotic-Antimycotic (1%, AA, Invitrogen) and cultured at 37 °C and 5% CO₂. For passaging, confluent cells were detached using Trypsin-EDTA 0.05% (Invitrogen) and re-seeded at a dilution of 1:5. Transfected CT26 cells were kept in the same culture conditions as CT26.wt cells.
1.2 Expression and Purification of Recombinant Human CAIX
Carbonic anhydrase IX (CAIX) was cloned in pCEP4 with a secretion sequence at its 5', and expressed in HEK293-ebna cells by transient gene expression. The transfection mix was assembled as follow: 1.25 µg of plasmid DNA, 3.75 µg polyethylenimine (PEI) for 10⁶ cells, with a cell density of 20 × 10⁶ cells/mL for 3 hours at 37 °C, 5% CO₂, 120 rpm. After high-density incubation, cells were diluted to a concentration of 10⁶ cells/mL and incubated for 6 days at 37 °C, 5% CO₂, 120 rpm. Cells were harvested by centrifugation (4500 rpm 30 min, 4 °C), and 1 mL (dry volume) of cOmplete His-tag purification Resin (Roche) was added to the filtered supernatant and incubated for 2 hours, 120 rpm at room temperature. Resin was washed with 300 mL of wash-buffer (imidazole 10mM, PBS/NaCl 250mM) and CAIX was eluted with 250 mM imidazole PBS/NaCl 250 mM. Elution fractions (1 mL) were loaded onto SDS-Page. CAIX-enriched fractions were pooled and dialyzed against PBS.

1.3 Surface Plasmon Resonance
Surface Plasmon Resonance (SPR) experiments were performed on a Biacore S200 instrument (GE Healthcare) at room temperature. For all measurements, CM5 chips (Series S) and flow buffer of filtered PBS pH 7.4 with DMSO (5% v/v) were used. Human CAIX was immobilized on the chip to 500 response units (RU) using EDC·HCl and NHS following manufacturer instructions. Serial dilutions of compounds 1 and 2 in running buffer at a flow rate of 20 µL min⁻¹ were used as analytes. After each cycle, the sensor surface was regenerated by a short treatment with DMSO (50% v/v) in PBS. Sensorgrams were solvent corrected and the binding kinetics were analyzed with the Biacore S200 evaluation software using a 1:1 Langmuir.

1.4 In Vitro Cytotoxicity Assay
SKRC-52 cells were seeded in 96-well plates in RPMI added with 10% FCS (100 µL) at a density of 5 × 10³ cells per well and allowed to grow for
24 hours. The medium was replaced with medium containing different concentrations of test substance (1:3 dilution steps) and plates were incubated under standard culture conditions. After 72 hours the medium was removed, MTS cell viability dye (20 µL, Promega) in 150 µL of the medium was added, the plates were incubated for 2 hours under culture conditions and the absorbance at 490 nm measured on a Spectra Max Paradigm multimode plate reader (Molecular Devices; background correction was performed by measuring the absorbance at 630 nm). Experiments were performed in triplicate and average cell viability calculated as measured background corrected absorbance divided by the absorbance of untreated control wells. IC\textsubscript{50} values were determined by fitting data to the four-parameter logistic equation, using a Prism 6 software (GraphPad Software) for data analysis.

1.5 FACS analysis

For cellular expression analysis of human CAIX, cells were detached with 50 mM EDTA and 5 × 10\textsuperscript{5} cells were stained with an anti-CAIX specific antibody (PBH11 raised by Phage Display Technology, Philogen) (500 nM, 1 hour, 4 °C) in a volume of 100 µl FACS-Buffer (0.5% BSA, 2 mM EDTA in PBS). For signal amplification, the anti-CAIX antibody was detected with an anti-human AlexaFluor647 labeled antibody (Invitrogen) (1:200, 45 min, 4 °C). In-between and after staining cells were washed with 100 µl FACS-Buffer and centrifuged at 1100 rpm for 3 min. Stained cells were analyzed with a 2L-Cytoflex (Beckman-Coulter). SKRC-52 cells were used as positive control and CT26.wt cells as negative control. Results were analyzed with FlowJo 9 (FlowJo LLC).

1.6 Transfection of human CAIX in CT26 tumor cells and monoclonal selection

The gene for human CAIX was cloned into the mammalian expression vector pcDNA3.1(+) containing an antibiotic resistance for G418 Geneticin. 6 × 10\textsuperscript{7} CT26.wt cells were transfected with 60 µg of pcDNA3.1(+) containing the human CAIX gene using the AmaxaTM 4D-
Nucleofector (Lonza) with the SG Cell Line 4D-Nucleofector® X Kit L (Lonza) and re-seeded in complete growing medium. Three days after the transfection, the medium was replaced with RPMI (10% FCS, 1% AA) containing 0.5 mg/mL G418 (Merck) to select a stably transfected polyclonal cell line. To yield a monoclonal cell line, the stable cell line was stained as described for FACS analysis and single cell sorting was performed using a BD FACSaria III. Different clones were expanded and checked for antigen expression. Clone CT26.3E10 was selected for CAIX expression by FACS, immunofluorescence and confocal microscopic, and used for further in vivo experiments.

1.7 Animal Studies
All animal experiments were conducted in accordance with Swiss animal welfare laws and regulations under the license number 27/2015 granted by the Veterinäramt des Kantons Zürich.

1.8 Implantation of Subcutaneous Tumors
SKRC-52 cells were grown to 80% confluence and detached with Trypsin-EDTA 0.05% (Life Technologies). Cells were washed with Hank’s Balanced Salt Solution (HBSS, pH 7.4) once, counted and re-suspended in HBSS to a final concentration of $3.4 \times 10^7$ cells mL$^{-1}$. Aliquots of $5 \times 10^6$ cells (150 µL of a suspension) were injected subcutaneously in the right flank of female athymic BALB/c nu/nu mice (8-10 weeks of age, Janvier).

CT26.3E10 cells were grown to 80% confluence and detached with Trypsin-EDTA 0.05% (Life Technologies). Cells were washed with Hank’s Balanced Salt Solution (HBSS, pH 7.4) once, counted and re-suspended in HBSS to a final concentration of $6.0 \times 10^7$ cells mL$^{-1}$. Aliquots of $6 \times 10^6$ cells (100 µL of a suspension) were injected subcutaneously in the right flank of female BALB/c mice (8-10 weeks of age, Janvier).
1.9 IVIS Imaging

Female BALB/c mice bearing subcutaneous CT26.3E10 tumors were injected intravenously with acetazolamide labeled with the near infrared dye moiety IRDye680RD (AAZ-IRDye680RD; compound 6; 250 nmol/Kg), dissolved in sterile PBS (100 µL). Mice were anesthetized with isoflurane and fluorescence images acquired on an IVIS Spectrum imaging system (Xenogen, exposure 1s, binning factor 8, excitation at 675 nm, emission filter at 720 nm, f number 2, field of view 13.1). Images were taken before the injection and after 5 min, 1, 3 and 6 hours. Food and water was given ad libitum during that period. Mice were sacrificed after the last picture by cervical dislocation and organs were extracted and imaged using the indicated parameters.

1.10 Radiolabelling and Quantitative Biodistribution Studies

Radiolabeling procedures with technetium-99m were performed following described procedures [88, 132]. Briefly, compounds 7 and 8 (60 nmol) in TBS pH 7.4 (50 µl) were separately mixed with SnCl₂ (Sigma Aldrich, 200 µg) and sodium glucoheptonate (TCI, 20 mg) in H₂O (150 µl). Tris-buffered saline at pH 7.4 (600 µl) was added and the resulting solution degassed for 5 min by bubbling with nitrogen gas. The eluate from a ⁹⁹ᵐTc-generator (200 µl, ca. 200 MBq, Mallinckrodt) was added and the reaction mixture heated to 90 °C for 20 min. After cooling to room temperature, analytic aliquots were analyzed by RP-HPLC (XTerra C18, 5% MeCN in 0.1% aq. TFA to 80% over 20 min on a Merck-Hitachi D-7000 HPLC system equipped with a Raytest Gabi Star radiodetector) to determine radiolabelling yields. Technetium-99m incorporations >95% were routinely achieved.

SKRC-52 xenografted tumors were implanted into female BALB/c nu/nu mice (Janvier) as described above, and allowed to grow for three weeks to an average volume of 0.25 mL. Mice were randomized (n = 3 per group) and injected intravenously with radiolabelled preparations of compounds 7 or 8 (1-3 MBq, 30 nmol/Kg). Mice were sacrificed 6 hours after the injection by CO₂ asphyxiation and organs extracted, weighted
and radioactivity measured with a Packard Cobra γ-counter. Values are expressed in chapter IV as %ID/g ± SD.

The tumor targeting performance of L19-IL2 in SKRC-52 bearing nude mice was evaluated by quantitative biodistribution analysis of the radioiodinated protein as described previously [229]. Briefly, radioiodiated protein was injected into the lateral tail vein of mice (n = 3). The animals were sacrificed after 2 hours, organs were excised and radioactivity was counted in Cobra gamma counter (Packard). Values are expressed in chapter IV as %ID/g ± SD.

1.11 Therapy Experiments
SKRC-52 or CT26.3E10 tumors were implanted into female BALB/c mice (Janvier) as described above, and allowed to grow to an average volume of 100 mm³. Mice were randomly assigned into therapy groups of 4 or 5 animals and treatment started by injecting a solution of AAZ-ValCit-MMAE (compound 1), AAZ⁺-ValCit-MMAE (compound 2), untargeted control (compound 3), L19-IL2, combination or vehicle (PBS containing 1% of DMSO) intravenously (lateral tail vein) at the doses and with the schedules indicated in the text and in chapter IV. Compounds 1, 2 and 3 were injected as solutions in sterile PBS containing 1% DMSO. L19-IL2 was injected as solution in appropriate sterile formulation buffer (Phologen). Animals were weighed and tumor sizes measured daily with an electronic caliper. The tumor volume was calculated according to the formula (long side) × (short side) × (short side) × 0.5. Animals were sacrificed when the termination criteria were reached. Prism 6 software (GraphPad Software) was used for data analysis (regular two-way ANOVA with the Bonferroni test).

1.12 Histology, immunofluorescence and immunohistochemistry studies
SKRC-52 tumors were excised, snap-frozen in OCT medium (Thermo Scientific) and stored at -80 °C. Cryostat sections (7 µm) were cut, fixed with acetone and stained with hematoxylin and eosin (H&E) using routine
methods or subjected to immunofluorescence. Primary staining for NK cells was performed with a rat anti-murine NKp46 IgG (NCR1; Biolegend). Primary staining for apoptosis was performed with a rabbit anti-caspase 3 IgG (C8487; Sigma Aldrich). Donkey anti-rat IgG-AlexaFluor594 and goat anti-rabbit IgG-AlexaFluor488 (Molecular Probes by Life Technologies) were then used for microscopic detection as secondary antibodies. Double im-munohistochemistry was applied to frozen tumor sections to detect neutrophils (anti-myeloperoxidase rabbit polyclonal antibody, A0398, Dako-Agilent Technologies, Denmark; 1:1000) and macrophages (anti-CD68 rabbit polyclonal antibody, ab125212, Abcam, Cambridge, UK; 1:200). The reaction was visualized using 3-amino-9-ethylcarbazole (AEC) and 3,3′-diaminobenzidine (DAB) as chromogens for neutrophils and macrophages respectively, followed by light counterstain with hematoxylin).

Images were obtained using an Axioskop2 mot plus microscope (Zeiss) or the NDP.view2 soft-ware (Hamamatsu Photonics K.K., Japan), after the slides were scanned using a digital slide scanner (NanoZoomer-XR C12000, Hamamatsu Photonics K.K.).

For further evaluation of human CAIX expression $10^5$ cells were cultured overnight on a sterile covers slip. Cells were fixed with ice-cold acetone, blocked with PBS (2% BSA) and stained with 5 µg/mL the human anti-CAIX PBH11 in PBS (1% BSA). Detection was performed with an anti-human AlexaFluor488 labeled antibody (1:200 in PBS containing 1% BSA). The cytoskeleton was stained with Phalloidin-AlexaFluor647 (ThermoFisher) and cell nuclei were stained with DAPI (MolecularProbes). Microscopic analysis was performed with an Axioskop 2 mot plus microscope (Zeiss).

To determine CAIX internalization after transfection, CT26.3E10 cells were seeded into 4-well cover slip chamber plates (Sarstedt) at a density of $10^4$ cells per well in RPMI medium (1 mL, Invitrogen) supplemented with 10% FCS, AA and HEPES (10 mM) and allowed to grow for 24 hours under standard culture conditions. The medium was replaced with medium containing AAZ-Alexa594 (compound 5; 120 nM), after 1 hour Hoechst 33342 nuclear dye (Invitrogen) was added and randomly
selected colonies imaged on a SP8 confocal microscope equipped with an AOBS device (Leica Microsystems).

2. Chemical Procedures

2.1 General Chemical Procedures

Anhydrous solvents were purchased from Acros or Fluka. Peptide grade N,N-dimethylformamide (DMF) for solid phase synthesis was bought from ABCR. All other solvents were used as supplied by Fisher Chemicals, Merck or Sigma Aldrich in HPLC or analytical grade. H-Cys(Trt)-2-CT-polystyrene resin was purchased from RAPP Polymere. Maleimidocaproyl-ValCit-p-aminobenzylalcohol-MMAE and free MMAE were purchased from Levena Biopharma (No.9 Wei-di Road, Qixia District, Nanjing, 210046, China). Maleimidoethyl-IRDye680RD was purchased from LI-COR Biosciences (4647 Superior Street Lincoln, Nebraska USA 68504-5000). Ultra-Technekow DTE Mo-99/Tc-99m Generator was purchased from Mallinckrodt Pharmaceuticals. L19-IL2 was produced by Philogen S.p.A. (Via Bellaria, 35, 53018 Sovicille SI, Italy) and diluted to the concentration used for therapy studies with the appropriate formulation buffer (Philogen). All other reagents were purchased from Sigma Aldrich, Acros, ABCR or TCI and used as supplied. All reactions using anhydrous conditions were performed using oven-dried glassware under an atmosphere of argon. Silica for flash column chromatography (high-purity grade, pore size 60 Å) was purchased from Sigma Aldrich. Concentration under reduced pressure was performed by rotary evaporation at 40 °C at the appropriate pressure. Yields refer to chromatographically purified and spectroscopically pure compounds, unless noted otherwise.

High-Resolution Mass Spectrometry (HRMS) spectra and analytical Reversed-Phase Ultra Performance Liquid Chromatography (UPLC) were recorded on a Waters Xevo G2-XS QTOF coupled to a Waters Acquity UPLC H-Class System with PDA UV detector, using a ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 μm, 2.1 mm × 50 mm at a flow rate of 0.6
mL min\(^{-1}\) with linear gradients of solvents A and B (A = Millipore water with 0.1% FA, B = MeCN with 0.1% FA). Preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) were performed on a Waters Alliance HT RP-HPLC with PDA UV detector, using a Synergi 4µm, Polar-RP 80Å 10 × 150 mm C18 column at a flow rate of 4 mL min\(^{-1}\) with linear gradients of solvents A and B (A = Millipore water with 0.1% TFA, B = MeCN with 0.1% TFA).

2.2 Peptide Synthesis

Peptidic precursors of compounds 1, 2, 3, 5 and 6, as well as compounds 4, 7 and 8 were synthesized by solid phase peptide synthesis (SPPS), using Fmoc-protected amino acids. Suitable resins (300-500 mg) were swollen with DMF (10 mL) for 15 min inside a syringe equipped with a filter pad. Fmoc deprotection was achieved by shaking the resin with 20% v/v piperidine in DMF (3 × 5 mL × 10 min). The appropriate Fmoc-protected amino acid (3 eq) was activated with HATU (3 eq) and DIPEA (6 eq) in DMF (5 mL) for 15 min at 0 °C. After this time the solution was allowed to react with the resin for 1 hour, discarded. Couplings and deprotections were alternated with DMF washing steps (4 × 10 mL), until the desired peptide sequence was obtained on solid phase.

Alkynes bearing acetazolamide or a free amide were then reacted by CuAAC “click” reaction with the corresponding peptide derivatives (carrying an azide moiety) on the resin. Side chain deprotection, cleavage from the resin, treatment with TCEP (30 eq) and RP-HPLC purification allowed to yield pure peptides, as confirmed by LC/MS analysis.
2.3 Chemical Synthesis of Present Compounds

AAZ-ValCit-MMAE – 1

Chemical Formula: C_{98}H_{151}N_{25}O_{28}S_{3}
Molecular Weight: 2223.61

Compound 1 was prepared according to previously described procedures [132].
**AAZ***-ValCit-MMAE – 2*

Chemical Formula: C$_{124}$H$_{180}$N$_{28}$O$_{37}$S$_3$

Molecular Weight: 2751.14

Compound 10 (3.5 mg, 2.44 µmol, 1.1 eq) was dissolved in degassed PBS (pH 7.4; 600 µl). Commercially available Maleimidocaproyl-ValCit-p-aminobenzylalcohol-MMAE (3.0 mg, 2.23 µmol, 1.0 eq) was added as a DMF solution (500 µl) and the mixture was stirred at room temperature. After 3 hours, UPLC-MS indicated completion. The solvents were removed under vacuum and the crude was diluted in a 1:1 H$_2$O/MeCN mixture (1 mL). The mixture was purified by RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). Pure fractions were collected and lyophilized overnight to achieve 2 as a white solid (3.1 mg; 1.13 µmol; 50.8% yield).

HRMS: ($m$/z) [M+2H]$^{2+}$ C$_{124}$H$_{182}$N$_{28}$O$_{37}$S$_3$, 1375.6113, found 1375.6986; [M+3H]$^{3+}$ C$_{124}$H$_{183}$N$_{28}$O$_{37}$S$_3$, 917.4075, found 917.4496.
Figure IX.1 Analytical UPLC trace of targeted conjugate 2 on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
Chemical Formula: C_{122}H_{179}N_{25}O_{35}S
Molecular Weight: 2587.97

Compound 11 (2.5 mg, 1.97 µmol, 1.3 eq) was dissolved in degassed PBS (pH 7.4; 600 µl). Commercially available Maleimidocaproyl-ValCit-p-aminobenzylalcohol-MMAE (2.0 mg, 1.52 µmol, 1.0 eq) was added as a DMF solution (500 µl) and the mixture was stirred at room temperature. After 3 hours, UPLC-MS indicated completion. The solvents were removed under vacuum and the crude was diluted in a 1:1 H_2O/MeCN mixture (1 mL). The mixture was purified by RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). Pure fractions were collected and lyophilized overnight to achieve 2 as a white solid (2.2 mg; 0.85 µmol; 56.4% yield).

HRMS: (m/z) [M+2H]^{2+} C_{122}H_{181}N_{25}O_{36}S, 1294.1358, found 1294.2177; [M+3H]^{3+} C_{122}H_{182}N_{25}O_{36}S, 863.0905, found 863.1285.
Figure IX.2 Analytical UPLC trace of targeted conjugate 3 on a BEH C18 Column, 130 Å, 1.7 μm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
AAZ$^+\text{-NH}_2$ ligand – 4

Chemical Formula: C$_{45}$H$_{62}$N$_{12}$O$_{15}$S$_2$
Molecular Weight: 1075.18

Compound 4 was prepared according to previously described procedures [63].

AAZ-Alexa594 – 5

Chemical Formula: C$_{79}$H$_{91}$N$_{18}$O$_{25}$S$_5$
Molecular Weight: 1792.94

Compound 5 was prepared according to previously described procedures [132].
**AAZ-IRDye680RD – 6**

Chemical Formula: $\text{C}_{75}\text{H}_{97}\text{ClN}_{19}\text{O}_{25}\text{S}_{6}$
Molecular Weight: 1892.52

Compound 6 was prepared according to previously described procedures [132].

**AAZ-$^{99m}\text{Tc}$ chelator – 7**

Chemical Formula: $\text{C}_{30}\text{H}_{46}\text{N}_{12}\text{O}_{13}\text{S}_{3}$
Molecular Weight: 878.95

Compound 7 was prepared according to previously described procedures [88, 132].
Chemical Formula: C_{52}H_{70}N_{14}O_{19}S_{3}
Molecular Weight: 1291.39

Commercially available pre-loaded Fmoc-Cys(Trt)-OH on polystyrene resin (500 mg, 0.32 mmol) was swollen in DMF for 15 min. The Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 mL) and the resin washed with DMF (4 × 10 min × 10 mL). Fmoc-Asp(OtBu)-OH (388 mg, 0.95 mmol, 3 eq) was activated with HATU (358 mg, 0.95 mmol, 3 eq), and DIPEA (328 µL, 1.88 mmol, 6 eq) in DMF (5 mL) at 0 °C for 5 min and then reacted with the resin for 1 h under gentle agitation. After washing the resin with DMF (4 × 10 min × 10 mL) the Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 mL) and the resin washed with DMF (4 × 10 min × 10 mL) before the peptide was extended with Boc-Lys(Fmoc)-OH (445 mg, 0.95 mmol, 3 eq), Fmoc-Lys(N3)-OH (374 mg, 0.95 mmol, 3 eq), Fmoc-Asp(OH)-OtBu (388 mg, 0.95 mmol, 3 eq), Fmoc-Asp(OH)-OtBu (388 mg, 0.95 mmol, 3 eq) and 4,4-bis(4-hydroxyphenyl)valeric acid (162 mg, 0.95 eq, 3 eq) in the indicated order using the same coupling conditions (HATU/DIPEA), Fmoc-deprotection (20% piperidine in DMF) and washing step with DMF mentioned before.
After the last peptide-coupling step, a solution of CuI (18 mg, 0.095 mmol, 0.3 eq), TBTA (16 mg, 0.031 mmol, 0.1 eq) and N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)hex-5-ynamide (261 mg, 0.95 mmol, 3 eq) in a mixture of DMF (2.5 mL) and THF (2.5 mL) was prepared and reacted with the resin at room temperature for 48 h. After washing with DMF (4 × 10 min × 10 mL), EDTA 50 mM (4 × 10 min × 10 mL) and DCM (4 × 10 min × 10 mL), the compound was cleaved from the resin by agitating with a mixture of TFA (8.6 mL), TIS (1.6 mL), H₂O (400 µL), m-Cresol (400 µL) and Thioanisol (400 µL) at room temperature for 1 h. Cleavage solution was added drop-wise to ice cold diethyl ether (50 mL) obtaining a white precipitate. The pellet was collected by centrifugation, dried under vacuum, redissolved in Millipore water and added with an excess of Tris(2-carboxyethyl)phosphine hydrochloride (30 eq). The product was purified by reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). After lyophilization the final compound was collected as a white powder (91 mg, 70.5 µmol, 22% yield).

HRMS: (m/z) [M+H]+ C₅₂H₇₁N₁₄O₁₉S₃, 1291.4104, found 1291.4219.

Figure IX.3 Analytical HPLC trace of compound 8 on a Synergi RP Polar column at a flow rate of 4 mL min⁻¹, 5% MeCN in 0.1% aq. TFA to 80% MeCN in 20 min. Please disregard as an artifact the injection peak at around 2 minutes.
AAZ-SMDC precursor – 9

Chemical Formula: C_{30}H_{46}N_{14}O_{13}S_{3}
Molecular Weight: 906.96

Compound 7 was prepared according to a previously described method [49].
Commercially available pre-loaded Fmoc-Cys(Trt)-OH on polystyrene resin (300 mg, 0.19 mmol) was swollen in DMF for 15 min. The Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 mL) and the resin washed with DMF (4 × 10 min × 10 mL). Fmoc-Asp(OtBu)-OH (233 mg, 0.57 mmol, 3 eq) was activated with HATU (215 mg, 0.57 mmol, 3 eq), and DIPEA (197 µL, 1.13 mmol, 6 eq) in DMF (3 mL) at 0 °C for 15 min and then reacted with the resin for 1 hour under gentle agitation. After washing the resin with DMF (4 × 10 min × 10 mL) the Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 mL) and the resin washed with DMF (4 × 10 min × 10 mL) before the peptide was extended with Fmoc-Arg(Pbf)-OH (368 mg, 0.57 mmol, 3 eq), Fmoc-Asp(OtBu)-OH (233 mg, 0.57 mmol, 3 eq), Fmoc-Lys(N3)-OH (224 mg, 0.57 mmol, 3 eq), Fmoc-Asp-OtBu (233, 0.57 mmol, 3 eq), Fmoc-Asp-OtBu (233, 0.57 mmol, 3 eq) and 4,4-bis(4-hydroxyphenyl)valeric acid (162 mg, 0.57 eq, 3 eq) in the indicated order using the same coupling conditions (HATU/DIPEA), Fmoc-deprotection (20% piperidine in DMF) and washing step with DMF mentioned before.
After the last peptide-coupling step, a solution of CuI (11 mg, 0.06 mmol, 0.3 eq), TBTA (10 mg, 0.02 mmol, 0.1 eq) and N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)hex-5-ynamide (155 mg, 0.57 mmol, 3 eq) in a mixture of DMF (1.5 mL) and THF (1.5 mL) was prepared and reacted with the resin at room temperature for 48 h. After washing with DMF (4 × 10 min × 10 mL), EDTA 50 mM (4 × 10 min × 10 mL) and DCM (4 × 10 min × 10 mL), the compound was cleaved from the resin by agitating with a mixture of TFA (6 mL), TIS (1.1 mL), H₂O (300 µL), m-Cresol (300 µL) and Thioanisol (300 µL) at room temperature for 1 h. Cleavage solution was added drop-wise to ice cold diethyl ether (50 mL) obtaining a white precipitate. The pellet was collected by centrifugation, dried under vacuum, redissolved in Millipore water and added with an excess of Tris(2-carboxyethyl)phosphine hydrochloride (30 eq). The product was purified by reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). After lyophilization the final compound was collected as a white powder (25 mg, 17.4 µmol, 9% yield).

HRMS: (m/z) [M+H]⁺ C₅₆H₇₆N₁₇O₂₂S₃, 1434.4435, found 1434.4501; [M+2H]²⁺ C₅₆H₇₇N₁₇O₂₂S₃, 717.7218, found 717.7317.

Figure IX.4 Analytical UPLC trace of compound 10 on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
Commercially available pre-loaded Fmoc-Cys(Trt)-OH on polystyrene resin (300 mg, 0.19 mmol) was swollen in DMF for 15 min. The Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 mL) and the resin washed with DMF (4 × 10 min × 10 mL). Fmoc-Asp(OtBu)-OH (233 mg, 0.57 mmol, 3 eq) was activated with HATU (215 mg, 0.57 mmol, 3 eq), and DIPEA (197 µL, 1.13 mmol, 6 eq) in DMF (3 mL) at 0 °C for 15 min and then reacted with the resin for 1 h under gentle agitation. After washing the resin with DMF (4 × 10 min × 10 mL) the Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 mL) and the resin washed with DMF (4 × 10 min × 10 mL) before the peptide was extended with Fmoc-Arg(Pbf)-OH (368 mg, 0.57 mmol, 3 eq), Fmoc-Asp(OtBu)-OH (233 mg, 0.57 mmol, 3 eq), Fmoc-Lys(N3)-OH (224 mg, 0.57 mmol, 3 eq), Fmoc-Asp-OtBu (233, 0.57 mmol, 3 eq), Fmoc-Asp-OtBu (233, 0.57 mmol, 3 eq) and 4,4-bis(4-hydroxyphenyl)valeric acid (162 mg, 0.57 eq, 3 eq) in the indicated order using the same coupling
conditions (HATU/DIPEA), Fmoc-deprotection (20% piperidine in DMF) and washing step with DMF mentioned before. After the last peptide-coupling step, a solution of Cul (11 mg, 0.06 mmol, 0.3 eq), TBTA (10 mg, 0.02 mmol, 0.1 eq) and Hex-5-ynamide (63 mg, 0.57 mmol, 3 eq) in a mixture of DMF (1.5 mL) and THF (1.5 mL) was prepared and reacted with the resin at room temperature for 48 h. After washing with DMF (4 × 10 min × 10 mL), EDTA 50 mM (4 × 10 min × 10 mL) and DCM (4 × 10 min × 10 mL), the compound was cleaved from the resin by agitating with a mixture of TFA (6 mL), TIS (1.1 mL), H$_2$O (300 µL), m-Cresol (300 µL) and Thioanisol (300 µL) at room temperature for 1 hour. Cleavage solution was added drop-wise to ice cold diethyl ether (50 mL) obtaining a white precipitate. The pellet was collected by centrifugation, dried under vacuum, redissolved in Millipore water and added with an excess of Tris(2-carboxyethyl)phosphine hydrochloride (30 eq). The product was purified by reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). After lyophilization the final compound was collected as a white powder (27 mg, 21.2 µmol, 11% yield).

HRMS: ($m/z$) [M+H]$^+$ C$_{54}$H$_{76}$N$_{14}$O$_{20}$S, 1271.4924, found 1271.5669; [M+2H]$^{2+}$ C$_{54}$H$_{76}$N$_{14}$O$_{20}$S, 636.2462, found 636.2623.

**Figure IX.5** Analytical UPLC trace of compound 11 on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min$^{-1}$, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
3. Statistical analysis of therapy experiment

Differences in tumor volume between therapeutic groups were compared using the two-way ANOVA analysis with Bonferroni post-test of Graphpad Prism 6 (La Jolla, CA, USA).

Therapy experiment 1 – Comparison between AAZ⁺-ValCit-MMAE and AAZ-ValCit-MMAE in SKRC-52 bearing nude mice

Tumor Size (mm³)

Vehicle vs. Compound 2 (AAZ⁺-ValCit-MMAE):

- day 15  p < 0.05
- day 16  p < 0.01
- day 17  p < 0.001
- from day 18  p < 0.0001

Vehicle vs. Compound 3 (negative control):

- non-significant differences

Vehicle vs. “presaturation” group:

- day 16  p < 0.01
- day 17  p < 0.01
- from day 18  p < 0.0001

Vehicle vs. Compound 1 (AAZ-ValCit-MMAE):

- day 14  p < 0.05
- day 15  p < 0.05
- day 16  p < 0.001
- from day 17  p < 0.0001

Compound 2 (AAZ⁺-ValCit-MMAE) vs. Compound 3 (negative control):

- day 18  p < 0.05
- day 19  p < 0.05
- day 20  p < 0.01
- from day 21  p < 0.0001
Compound 2 (AAZ⁺-ValCit-MMAE) vs. “presaturation” group:
 day 21 p < 0.01
 day 22 p < 0.001
 day 23 p < 0.001
 day 24 p < 0.001
 from day 25 p < 0.0001

Compound 1 (AAZ-ValCit-MMAE) vs. Compound 2 (AAZ⁺-ValCit-MMAE):
 day 28 p < 0.01

Compound 3 (negative control) vs. “presaturation” group:
 from day 24 p < 0.05

Compound 1 (AAZ-ValCit-MMAE) vs. compound 3 (negative control):
 day 20 p < 0.05
 day 21 p < 0.01
 day 22 p < 0.01
 day 24 p < 0.001
 from day 25 p < 0.0001

Compound 1 (AAZ-ValCit-MMAE) vs. “presaturation” group:
 day 25 p < 0.01
 day 26 p < 0.0001
 day 28 p < 0.01

**Body Weight Change (%)**

Vehicle vs. Compound 2 (AAZ⁺-ValCit-MMAE):
 day 24 p < 0.01
 from day 25 p < 0.001

Vehicle vs. Compound 3 (negative control):
 non-significant differences
Vehicle vs. “presaturation” group:

day 24  p < 0.05  

day 25  p < 0.05  

day 26  p < 0.0001  

Vehicle vs. Compound 1 (AAZ-ValCit-MMAE):

day 25  p < 0.05  

day 26  p < 0.001  

Compound 2 (AAZ*-ValCit-MMAE) vs. Compound 3 (negative control):

day 24  p < 0.05  

day 25  p < 0.0001  

Compound 2 (AAZ*-ValCit-MMAE) vs. “presaturation” group:

non-significant differences  

Compound 1 (AAZ-ValCit-MMAE) vs. Compound 2 (AAZ*-ValCit-MMAE):

day 26  p < 0.05  

day 28  non-significant difference  

Compound 3 (negative control) vs. “presaturation” group:

day 25  p < 0.05  

Compound 1 (AAZ-ValCit-MMAE) vs. compound 3 (negative control):

non-significant differences  

Compound 1 (AAZ-ValCit-MMAE) vs. “presaturation” group:

non-significant differences
Therapy experiment 2 – Effect of the combination AAZ+ ValCit-MMAE/L19-IL2 in SKRC-52 bearing nude mice

Tumor Size (mm³)
Vehicle vs. L19-IL2:
day 13 p < 0.05
from day 14 p < 0.0001

Vehicle vs. Compound 2 (AAZ+ ValCit-MMAE):
day 11 p < 0.01
from day 12 p < 0.0001

Vehicle vs. combination group:
day 11 p < 0.001
from day 12 p < 0.0001

L19-IL2 vs. Compound 2 (AAZ+ ValCit-MMAE):
day 21 p < 0.01
day 22 p < 0.001

L19-IL2 vs. combination group:
day 16 p < 0.05
day 17 p < 0.001
from day 18 p < 0.0001

Compound 2 (AAZ+ ValCit-MMAE) vs. combination group:
day 14 p < 0.05
from day 15 p < 0.0001

Body Weight Change (%)
Vehicle vs. L19-IL2:
day 16 p < 0.05
from day 17 non-significant difference
Vehicle vs. Compound 2 (AAZ⁺-ValCit-MMAE):  
non-significant differences

Vehicle vs. combination group:  
non-significant differences

L19-IL2 vs. Compound 2 (AAZ⁺-ValCit-MMAE):  
non-significant differences

L19-IL2 vs. combination group:  
non-significant differences

Compound 2 (AAZ⁺-ValCit-MMAE) vs. combination group:  
non-significant differences

Therapy experiment 3 – Effect of the combination AAZ⁺-ValCit-MMAE/L19-IL2 in CT26.3E10 bearing immunocompetent mice

Tumor Size (mm³)

Vehicle vs. L19-IL2:
day 18 \( p < 0.05 \)  
from day 20 \( p < 0.0001 \)

Vehicle vs. Compound 2 (AAZ⁺-ValCit-MMAE):
day 18 \( p < 0.05 \)  
day 20 \( p < 0.0001 \)  
day 21 \( p < 0.001 \)  
from day 22 \( p < 0.0001 \)

Vehicle vs. combination group:
day 17 \( p < 0.01 \)  
from day 18 \( p < 0.001 \)  
from day 20 \( p < 0.0001 \)

L19-IL2 vs. Compound 2 (AAZ⁺-ValCit-MMAE):
day 30  p < 0.01

Compound 2 (AAZ⁺-ValCit-MMAE) vs. combination group:
day 29  p < 0.05
day 30  p < 0.05
day 31  p < 0.01

L19-IL2 vs. combination group:
day 28  p < 0.001
day 29  p < 0.001
day 30  p < 0.0001

Body Weight Change (%)
Vehicle vs. L19-IL2:
non-significant differences

Vehicle vs. Compound 2 (AAZ⁺-ValCit-MMAE):
non-significant differences

Vehicle vs. combination group:
day 22  p < 0.05
from day 23  p < 0.0001

L19-IL2 vs. Compound 2 (AAZ⁺-ValCit-MMAE):
non-significant differences

L19-IL2 vs. combination group:
day 22  p < 0.05
day 23  p < 0.01
day 24  p < 0.01
day 25  p < 0.01
from day 28  p < 0.0001
Compound 2 (AAZ+-ValCit-MMAE) vs. combination group:
day 29 

p < 0.01
This section corresponds to the experimental part of our manuscript “Chemically-defined antibody- and small molecule-drug conjugates for in vivo targeting applications: a comparative analysis” by S. Cazzamalli, A. Dal Corso, F. Widmayer & Dario Neri. Published in the Journal of the American Chemical Society in 2018.

1. Biological Procedures

1.1 Isolation of XE114 single chain Fv against CAIX

120 pmol of biotinylated His-tagged CAIX (amino acids 120-397 of the full-length protein; see sequence below) were incubated with 60 µL of streptavidin-coated dynabeads (Invitrogen) for 30 minutes at room temperature with agitation. Unbound CAIX antigen was washed from the beads 3× with PBS pH 7.4. The antigen-bead complex was incubated with 10¹² transforming units (t.u.) of phage antibodies in 1 mL 2% milk in PBS for 1 hour with rotation at room temperature. Unbound phages were washed from the beads using 6× 1 mL PBST followed by 6× 1mL PBS. Bound phage were eluted from the beads by addition of 800 µL 100 mM TAE and incubation for 5 min. The eluted phage were immediately neutralized by the addition of 200 µL 1M TBS pH 7.4. Once eluted and neutralized, the phage were used to infect exponentially growing E. coli TG1 cells. Two rounds of panning were performed against His-tagged CAIX and the selection outputs were tested for binding to His-tagged CAIX in an ELISA.
Clones giving a positive signal in an ELISA were screened by BIACore to confirm which scFvs were able to bind to CAIX. Supernatant screening was performed on a Biacore 3000 instrument (Biacore). 2100 response units (RU) of recombinant His-tagged CAIX were immobilized onto a CM5 chip (Biacore). 15 µL of each supernatant was allowed to flow over the coated chip at a flow rate of 10 µL/min. Positive clones, including the anti-CAIX scFv antibody XE114, were identified and sequenced (sequenced using conventional methods to identify unique scFvs).

1.2 Cell Culture
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). SKRC-52 human renal cell carcinoma cells were obtained from our collaborator Professor E. Oosterwijk (Radbound University Nijmegen Medical Centre, Nijmegen, The Netherlands). Upon thawing, SKRC-52 cells were maintained in RPMI medium (Invitrogen) supplemented with fetal calf serum (10%, FCS, Invitrogen) and Antibiotic-Antimycotic (1%, AA, Invitrogen) at 37 °C and 5% CO2. For passaging, cells were detached using Trypsin-EDTA 0.05% (Invitrogen) when reaching 90% confluence and re-seeded at a dilution of 1:6.

1.3 Protein Production and Purification
The gene structure for IgG1(KSF) mutant, its cloning, expression and characterization have previously been described [34, 103]. The IgG1(XE114) antibody gene (sequence reported below) was cloned in pMM137 using strategies elsewhere described [230]. The protein was expressed in CHO-S cells (Invitrogen) by transient gene expression. Briefly, CHO cells in suspension were first counted and re-suspended in fresh ProCHO medium to a final cells concentration of $4 \times 10^6$ cells/mL. 0.9 µg/million cells DNA and 2.5 µg/million cells PEI were added carefully to the cells. Cells were incubated in a shaker at 31 °C × 150 rpm for 6
days. After incubation the suspension was centrifuged at 4 °C × 6500 rpm for 25 minutes (SLA-3000 rotor) using Sorvall RC 5C Plus centrifuge. Supernatant was harvested and filter by PD-10 column and loaded onto a protein A column. The column was thereafter washed with 200 mL of Buffer A (100 mM NaCl, 0.5 mM EDTA, 0.1% Tween 20 in PBS) and then with 200 mL Buffer B (500 mM NaCl, 0.5 mM EDTA in PBS). The antibody product was eluted using 10-15 mL 0.1 M glycine at pH = 3 and fractions of 1 mL were collected. OD at an absorbance of 280 nm (OD280) was measured and fractions containing protein (OD280 > 0.1 mg/mL) were pooled and loaded on SpectraPor dialysis membrane MW 12-14000 (Spectrum laboratories) and dialysed in PBS o/n at 4 °C. After dialysis, IgG1(XE114) was characterized by SDS-PAGE, size exclusion chromatography and mass spectrometry.

1.4 Protein Sequence of His-tag-CAIX
GDPQEPQNNAHKEDQKDDQSHWRYGDPWPRVSPACAGRFQSPV
DIRPQLAAFCPALRPLELLGFQLPPLPRLRLRNNGHSVQLTLPPGLEMA
LGPGREYRALQLHLHWGAAGRPGEHTVEGHRFPAPVHVHVLSTAFAR
VDEALGRPGGLAVLAACLEEGPEENSAYEQLLSRLEEIAESETVQVG
LDISALLPSSFSRYFQYEGSLTTPCAQGVIWTVFNQTVMLSAKQLHTL
SD-TLWPGDSRLQLNFRATQPLNGRVIEASFPAGVDSSHHHHH

1.5 Protein Sequence of IgG1(XE114)
The full amino acid sequences for IgG1(XE114) can be found here below. The cysteine residues of the heavy chain in position 219, 225 and 228, were mutated into serine residues, thus allowing site-specific conjugation of Vedotin to the IgG molecule. The asparagine residue of the light chain in position 88 was mutated into glutamine residue, in order to avoid glycosylation.
Heavy Chain
EVQLLESGGGLVQPGGLRLSCAASGFTSSYAMSWVRQAPGKGLEW
VSAIDGGSGSTYADVSKGRFTISRDNSKNTLYLQMNLRAEDTAVYYC
VKGPVFDYWGQTLVTSSASTKGPSVFPLPSSKSTGGTGALICL
VKDYFPEPVTSWSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSL
GTQTYICNVNHKPSNTKVDKKVEPKSSDTHTPSPPAPELLGGPSVFL
FPKPKDLMISRTPEVTVCVVDVSHEDPEVKFNWYVDGEVHNAKTK
PREEQYNYSTYRVSVLTVHQDWANGKEYCKVSNNKAPAPIEKTIASKA
KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAYEVESNQQP
ENNYKTTPVLDSGFLYLSKLTVDKSRWQQGQNFSCSVMHEALHN
HYTQKSLSLSPGK

Light Chain
SSELTQDPAVSVALQVTIRCTCQGDSLRSYASYWQQKPGQAPVLVI
Y GKNRPSGIPDRFGSGSSGNTASLTITGAQAEADEDAYYCQSSKWSWD
PVVFGGGTTLVGLQPKAAPSVTLPSSELQANKATLCLISDFYPG
AVTVAWKADSSPVKAGVETTTSPQKSNKYYAASSYLTSPTPEQWKSCHKS
YSCQVTHEGSTVEKTAPTECS

1.6 Antibody-MMAE Conjugates Preparation
IgG1(XE114) was first reduced with 60 equivalents (per antibody molecule) of TCEP-HCl in PBS (pH = 7.4). After reduction, the protein was purified by FPLC using a HiPrep 26/10 Desalting Column (GE Healthcare). The collected fractions containing the product were pooled and the protein concentration was determined by measuring the OD280. Commercially available MC-ValCit-PAB-MMAE (10 equivalents) were dissolved in DMSO to obtain a final concentration of 10% (v/v) when added to the reduced protein. The mixture was stirred for 1 hour at RT and then it was quenched with L-Cys (Fluka) at a final concentration of 1 mM for 10 min at RT under stirring. The final product was purified by FPLC. Fraction containing the ADC(+) were pooled and the final protein concentration was determined by measuring the OD280. ADC(-) was prepared following procedures described in the literature [34].
1.7 Protein Characterization

1.7.1 SDS-PAGE

Protein samples were diluted to 0.2-0.3 mg/mL in PBS and mixed with either reducing or non-reducing 5× Loading buffer. Samples were denatured 5’ at 95 °C and loaded on NuPAGE 4-12% Bis-Tris Gel (Novex™ by Life Technologies). 1× MES NuPAGE (Novex™ by Life Technologies) was used as running buffer and electrophoresis was performed at 180 V, 110 mA for 1 hour. Gel was rinsed with deionized water and stained in Coomassie blue for 15-20 minutes on an orbital shaker. Staining solution was discarded and the gel was rinsed 3 times with deionized water and immerged in destaining solution (10% acetic acid/30% methanol/mQ water) for 3-12 hours on an orbital shaker. Destaining solution was discarded and recycled, gel was rinsed with deionized water and a picture of the gel was taken. Recipes for the 5× Loading buffer and Coomassie blue stain are as described in Table X.1 and X.2.

<table>
<thead>
<tr>
<th>100mL, 5X non-red Loading Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (250mM, pH 6.8)</td>
</tr>
<tr>
<td>Glycerol</td>
</tr>
<tr>
<td>SDS</td>
</tr>
<tr>
<td>Bromophenol blue</td>
</tr>
<tr>
<td>mQ water</td>
</tr>
</tbody>
</table>

**Table X.1** 5X Loading buffer recipe. For 5X reducing Loading buffer, add 5-10%(v/v) 2-mercaptoethanol.

<table>
<thead>
<tr>
<th>1L coomassie blue</th>
</tr>
</thead>
<tbody>
<tr>
<td>PlusOne Coomassie PhastGel Blue R-350</td>
</tr>
<tr>
<td>Methanol</td>
</tr>
<tr>
<td>Acetic Acid</td>
</tr>
<tr>
<td>mQ water</td>
</tr>
</tbody>
</table>

**Table X.2** Coomassie blue receipe
1.7.2 Gel Filtration Analysis

100 µL of diluted sample (final concentration 0.3-0.5 mg/mL) were loaded on FPLC (Äkta, GE Healthcare) and protein were separated by a Superdex200 10/300GL column (GE Healthcare) previously equilibrated with 1 CV PBS, using PBS as mobile phase at a flow rate of 0.5 mL/min (column pressure limit set at 1.5 MPa). Proteins were detected by an UV-detector at a wavelength of 280 nm.

1.7.3 Mass Spectrometry

Samples were diluted to about 0.1 mg/mL and LC-MS was performed on a Waters Xevo G2XS Qtof instrument (ESI-ToF-MS) coupled to a Waters Acquity UPLC H-Class System using a 2.1 × 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).

1.8 Animal Studies

All animal experiments were conducted in accordance with Swiss animal welfare laws and regulations under the license number 27/2015 granted by the Veterinäramt des Kantons Zürich.

1.9 Implantation of Subcutaneous Tumors

SKRC-52 cells were grown to 80% confluence and detached with Trypsin-EDTA 0.05% (Life Technologies). Cells were washed with Hank’s Balanced Salt Solution (HBSS, pH 7.4) once, counted and re-suspended in HBSS to a final concentration of 3.4 × 10⁷ cells mL⁻¹. Aliquots of 5 × 10⁶ cells (150 µL of the suspension) were injected subcutaneously in the right flank of female athymic BALB/c nu/nu mice (8-10 weeks of age, Janvier).
1.10 Tumor and Organ Penetration Studies

BALB/c nu/nu mice bearing established subcutaneous SKRC-52 tumors (200-300 mm³) were injected intravenously with anti-CAIX IgG1(XE114) mutant (300 µg in PBS pH 7.4 solution) in sterile PBS buffer pH 7.4 (200 µL). After 24 hours animals were sacrificed by CO₂ asphyxiation. Organs and tumors were extracted and flash-frozen in Neg-50 cryo medium (Thermo Fisher Scientific) using liquid nitrogen. After warming to -20 °C, samples were cut into sections of 10 µm width and stained with rabbit anti-human Fc (Molecular Probes by Life Technologies), to detect the antibody in IgG format. Nuclear staining was performed with DAPI. Anti-rabbit IgG-AlexaFluor488 (Molecular Probes by Life Technologies) was used as secondary antibody for microscopic detection. Alternatively, SKRC-52 bearing BALB/c nu/nu mice (200-300 mm³) were administered with Alexa594 (Thermo Fisher Scientific) labeled compounds 5 or 6 (30 nmol), which were dissolved in sterile PBS buffer pH 7.4 (150 µL). Animals were sacrificed after 1 hour and organs and tumors were processed as above stated. Sections of 10 µm width were obtained and nuclear staining was performed with DAPI. All pictures were acquired on an Axioskop 2 fluorescence microscope (Zeiss).

1.11 Quantitative Biodistribution Studies

The tumor targeting properties of AAZ⁺ were assessed by quantitative biodistribution analysis in SKRC-52 tumor bearing mice, following described procedures reported for similar compounds [88, 132]. Briefly, compound 3 (60 nmol) in TBS pH 7.4 (50 µL) was mixed with SnCl₂ (Sigma Aldrich, 200 µg) and sodium glucoheptonate (TCI, 20 mg) in H₂O (150 µL). Tris-buffered saline at pH 7.4 (600 µL) was added and the resulting solution degassed for 5 min by bubbling with nitrogen gas. The eluate from a ⁹⁹ᵐTc-generator (200 µL, ca. 200 MBq, Mallinckrodt) was added and the reaction mixture heated to 90 °C for 20 min. After cooling to room temperature, analytic aliquots were analyzed by RP-HPLC.
(XTerra C18, 5% MeCN in 0.1% aq. TFA to 80% over 20 min on a Merck-Hitachi D-7000 HPLC system equipped with a Raytest Gabi Star radiodetector) to determine radiolabelling yields. Technetium-99m incorporations >95% were routinely achieved. SKRC-52 tumor cells were implanted into female BALB/c nu/nu mice (Janvier) as described above, and allowed to grow for three weeks to an average volume of 250 mm$^3$. Mice were randomized ($n = 3$ per group) and injected intravenously with the radiolabelled preparation of SM(+)$^{99m}$Tc chelator (1-3 MBq, 30 nmol/Kg). Mice were sacrificed 6 hours after the injection by CO$_2$ asphyxiation and organs extracted, weighted and radioactivity measured with a Packard Cobra γ-counter. Values are expressed as %ID/g ± SD.

The tumor targeting properties of the anti-CAIX IgG1(XE114) antibody were assessed by quantitative biodistribution analysis in SKRC-52 tumor bearing mice. IgG1(XE114) was radioiodinated with $^{125}$I (PerkinElmer) and chloramine T (Sigma) and purified on a PD-10 column (GE Healthcare). Immunodeficient BALB/c nu/nu mice bearing subcutaneous established SKRC-52 xenografts ($n = 5$; average tumor volume of about 250 mm$^3$) were injected into the lateral tail vein at a dose of 5 mg/kg of the protein. The animals were sacrificed after 24 hours, organs were excised and radioactivity was counted in Cobra gamma counter (Packard). Values are expressed as %ID/g ± SD.

**1.12 Therapy Experiments**

SKRC-52 tumor cells were implanted into female BALB/c mice (Janvier) as described above, and allowed to grow to an average volume of 100 mm$^3$. Mice were randomly assigned into therapy groups of 5 animals and treatment started by injecting a solution of SMDC(+), SMDC(-), ADC(+), ADC(-) or vehicle (PBS containing 1% of DMSO) intravenously (lateral tail vein) at the doses and with the schedules indicated in chapter V (text and in figures V.5). SMDC(+) and SMDC(-) were injected as solutions in sterile PBS containing 1% DMSO. ADC products were injected as solution in PBS buffer. Animals were weighed and tumor sizes measured daily with an electronic caliper. The tumor volume was calculated according to the formula (long side) × (short side) × (short side) × 0.5.
Animals were sacrificed when the termination criteria were reached. Prism 6 software (GraphPad Software) was used for data analysis (regular two-way ANOVA followed by Bonferroni test).

2. Chemical Procedures

2.1 General Chemical Procedures
Anhydrous solvents were purchased from Acros or Fluka. Peptide grade N,N-dimethylformamide (DMF) for solid phase synthesis was bought from ABCR. All other solvents were used as supplied by Fisher Chemicals, Merck or Sigma Aldrich in HPLC or analytical grade. H-Cys(Trt)-2-CT-polystyrene resin was purchased from RAPP Polymere. Maleimidocaproyl-ValCit-p-aminobenzylalcohol-MMAE was purchased from Levena Biopharma (No.9 Wei-di Road, Qixia District, Nanjing, 210046, China). Alexa Fluor™ 594 C5 Maleimide and Alexa Fluor™ 594 NHS Ester were purchased from Thermo Fisher Scientific (168 Third Avenue Waltham, MA USA 02451). Ultra-Technekow DTE Mo-99/Tc-99m Generator was purchased from Mallinckrodt Pharmaceuticals. All other reagents were purchased from Sigma Aldrich, Acros, ABCR or TCI and used as supplied. All reactions using anhydrous conditions were performed using oven-dried glassware under an atmosphere of argon. Silica for flash column chromatography (high-purity grade, pore size 60 Å) was purchased from Sigma Aldrich. Concentration under reduced pressure was performed by rotary evaporation at 40 °C at the appropriate pressure. Yields refer to chromatographically purified and spectroscopically pure compounds, unless noted otherwise. High-Resolution Mass Spectrometry (HRMS) spectra and analytical Reversed-Phase Ultra Perfor-mance Liquid Chromatography (UPLC) were recorded on a Waters Xevo G2-XS QTOF coupled to a Waters Acquity UPLC H-Class System with PDA UV detector, using a ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹ with linear gradients of solvents A and B (A = Millipore water with 0.1% FA, B = MeCN with 0.1% FA). Preparative reversed-
phase high-pressure liquid chromatography (RP-HPLC) were performed on a Waters Alliance HT RP-HPLC with PDA UV detector, using a Synergi 4µm, Polar-RP 80Å 10 × 150 mm C18 column at a flow rate of 4 mL min\(^{-1}\) with linear gradients of solvents A and B (A = Millipore water with 0.1% TFA, B = MeCN with 0.1% TFA).

2.2 Synthetic Procedures and Characterizations

2.2.1 Synthesis of SMDC(+)

Compound 2

Chemical Formula: C\(_{56}\)H\(_{75}\)N\(_{17}\)O\(_{22}\)S\(_{3}\)
Molecular Weight: 1434.49 g/mol
Commercially available pre-loaded Fmoc-Cys(Trt)-OH on polystyrene resin (1, 300 mg, 0.19 mmol) was swollen in DMF for 15 min. The Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 mL) and the resin washed with DMF (4 × 10 min × 10 mL). Fmoc-Asp(OtBu)-OH (233 mg, 0.57 mmol, 3 eq) was activated with HATU (215 mg, 0.57 mmol, 3 eq), and DIPEA (197 µL, 1.13 mmol, 6 eq) in DMF (3 mL) at 0 °C for 15 min and then reacted with the resin for 1 h under gentle agitation. After washing the resin with DMF (4 × 10 min × 10 mL) the Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 mL) and the resin washed with DMF (4 × 10 min × 10 mL) before the peptide was extended with Fmoc-Arg(Pbf)-OH (368 mg, 0.57 mmol, 3 eq), Fmoc-Asp(OtBu)-OH (233 mg, 0.57 mmol, 3 eq), Fmoc-Lys(N3)-OH (224 mg, 0.57 mmol, 3 eq), Fmoc-Asp-OtBu (233, 0.57 mmol, 3 eq), Fmoc-Asp-OEtBu (233, 0.57 mmol, 3 eq) and 4,4-bis(4-hydroxyphenyl)valeric acid (162 mg, 0.57 eq, 3 eq) in the indicated order using the same coupling conditions (HATU/DIPEA), Fmoc-deprotection (20% piperidine in DMF) and washing step with DMF mentioned before.

After the last peptide-coupling step, a solution of CuI (11 mg, 0.06 mmol, 0.3 eq), TBTA (10 mg, 0.02 mmol, 0.1 eq) and N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)hex-5-ynamide (155 mg, 0.57 mmol, 3 eq) in a mixture of DMF (1.5 mL) and THF (1.5 mL) was prepared and reacted with the resin at room temperature for 48 h. After washing with DMF (4 × 10 min × 10 mL), EDTA 50 mM (4 × 10 min × 10 mL) and DCM (4 × 10 min × 10 mL), the compound was cleaved from the resin by agitating with a mixture of TFA (6 mL), TIS (1.1 mL), H₂O (300 µL), m-Cresol (300 µL) and Thioanisol (300 µL) at room temperature for 1 hour. Cleavage solution was added drop-wise to ice cold diethyl ether (50 mL) obtaining a white precipitate. The pellet was collected by centrifugation, dried under vacuum, redissolved in Millipore water and added with an excess of Tris(2-carboxyethyl)phosphine hydrochloride (30 eq). The product was purified by reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). After lyophilization the final compound 2 was collected as a white powder (25 mg, 17.4 µmol, 9% yield).
HRMS: (m/z) [M+H]^+ C_{56}H_{76}N_{17}O_{22}S_3, 1434.4435, found 1434.4501; [M+2H]^{2+} C_{56}H_{77}N_{17}O_{22}S_3, 717.7218, found 717.7317.

Figure XI.1 Analytical UPLC trace of compound 2 on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.

SMDC(+)

Chemical Formula: C_{124}H_{180}N_{28}O_{37}S_3
Molecular Weight: 2751.14 g/mol

Thiol 2 (3.5 mg, 2.44 µmol, 1.1 eq) was dissolved in degassed PBS (pH 7.4; 600 µL). Commercially available Maleimidocaproyl-ValCit-p-aminobenzylalcohol-MMAE (3.0 mg, 2.23 µmol, 1.0 eq) was added as a DMF solution (500 µl) and the mixture was stirred at room temperature. After 3 hours, UPLC-MS indicated completion. The solvents were removed under vacuum and the crude was diluted in a 1:1 H₂O/MeCN mixture (1 mL). The mixture was purified by RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). Pure fractions were collected and lyophilized overnight to achieve SMDC(+) as a white solid (3.1 mg; 1.13 µmol; 50.8% yield).
HRMS: (m/z) [M+2H]\(^2+\) \(\text{C}_{124}\text{H}_{182}\text{N}_{28}\text{O}_{37}\text{S}_{3}\), 1375.6186, found 1375.6222; [M+3H]\(^3+\) \(\text{C}_{124}\text{H}_{182}\text{N}_{28}\text{O}_{37}\text{S}_{3}\), 917.4148, found 917.4207.

**Figure XI.2** Analytical UPLC trace of SMDC(+) on a BEH C18 Column, 130 Å, 1.7 μm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min\(^{-1}\), 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
2.2.2 Synthesis of SMDC(-)

Commercially available pre-loaded Fmoc-Cys(Trt)-OH on polystyrene resin (1, 300 mg, 0.19 mmol) was swollen in DMF for 15 min. The Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 mL) and the resin washed with DMF (4 × 10 min × 10 mL). Fmoc-Asp(ObtBu)-OH (233 mg, 0.57 mmol, 3 eq) was activated with HATU (215 mg, 0.57 mmol, 3 eq), and DIPEA (197 µL, 1.13 mmol, 6 eq) in DMF (3 mL) at 0 °C for 15 min and then reacted with the resin for 1 hour under gentle
agitation. After washing the resin with DMF (4 × 10 min × 10 mL) the Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 mL) and the resin washed with DMF (4 × 10 min × 10 mL) before the peptide was extended with Fmoc-Arg(Pbf)-OH (368 mg, 0.57 mmol, 3 eq), Fmoc-Asp(OtBu)-OH (233 mg, 0.57 mmol, 3 eq), Fmoc-Lys(N3)-OH (224 mg, 0.57 mmol, 3 eq), Fmoc-Asp-OtBu (233, 0.57 mmol, 3 eq), Fmoc-Asp-OtBu (233, 0.57 mmol, 3 eq) and 4,4-bis(4-hydroxyphenyl)valeric acid (162 mg, 0.57 eq, 3 eq) in the indicated order using the same coupling conditions (HATU/DIPEA), Fmoc-deprotection (20% piperidine in DMF) and washing step with DMF mentioned before. After the last peptide-coupling step, a solution of CuI (11 mg, 0.06 mmol, 0.3 eq), TBTA (10 mg, 0.02 mmol, 0.1 eq) and Hex-5-ynamide (63 mg, 0.57 mmol, 3 eq) in a mixture of DMF (1.5 mL) and THF (1.5 mL) was prepared and reacted with the resin at room temperature for 48 hours. After washing with DMF (4 × 10 min × 10 mL), EDTA 50 mM (4 × 10 min × 10 mL) and DCM (4 × 10 min × 10 mL), the compound was cleaved from the resin by agitating with a mixture of TFA (6 mL), TIS (1.1 mL), H$_2$O (300 µL), m-Cresol (300 µL) and Thioanisol (300 µL) at room temperature for 1 hour. Cleavage solution was added drop-wise to ice cold diethyl ether (50 mL) obtaining a white precipitate. The pellet was collected by centrifugation, dried under vacuum, redissolved in Millipore water and added with an excess of Tris(2-carboxyethyl)phosphine hydrochloride (30 eq). The product was purified by reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). After lyophilization the final compound 3 was collected as a white powder (27 mg, 21.2 µmol, 11% yield).

HRMS: (m/z) [M+H]$^+$ C$_{54}$H$_{75}$N$_{14}$O$_{20}$S, 1271.4924, found 1271.5669; [M+2H]$^{2+}$ C$_{54}$H$_{76}$N$_{14}$O$_{20}$S, 636.2462, found 636.2623.
**Figure XI.3** Analytical UPLC trace of compound 3 on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.

SMDC(−)

Chemical Formula: C\textsubscript{122}H\textsubscript{179}N\textsubscript{25}O\textsubscript{35}S
Molecular Weight: 2587.97 g/mol

Thiol 3 (2.5 mg, 1.97 µmol, 1.3 eq) was dissolved in degassed PBS (pH 7.4; 600 µL). Commercially available Maleimidocaproyl-ValCit-p-aminobenzylalcohol-MMAE (2.0 mg, 1.52 µmol, 1.0 eq) was added as a DMF solution (500 µL) and the mixture was stirred at room temperature. After 3 hours, UPLC-MS indicated completion. The solvents were removed under vacuum and the crude was diluted in a 1:1 H\textsubscript{2}O/MeCN mixture (1 mL). The mixture was purified by RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). Pure fractions were collected and lyophilized overnight to achieve compound SMDC(−) as a white solid (2.2 mg; 0.85 µmol; 56.4% yield).
HRMS: (m/z) [M+2H]$^{2+}$ C$_{122}$H$_{181}$N$_{25}$O$_{36}$S, 1294.1358, found 1294.2177; [M+3H]$^{3+}$ C$_{122}$H$_{182}$N$_{25}$O$_{36}$S, 863.0905, found 863.1285.

Figure XI.4 Analytical UPLC trace of SMDC(-) on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min$^{-1}$, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
2.2.3 Synthesis of SM(+)–99mTc chelator

Chemical Formula: C_{52}H_{70}N_{14}O_{19}S_{3}
Molecular Weight: 1291.39 g/mol

Commercially available pre-loaded Fmoc-Cys(Trt)-OH on polystyrene resin (1, 500 mg, 0.32 mmol) was swollen in DMF for 15 min. The Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 mL) and the resin washed with DMF (4 × 10 min × 10 mL). Fmoc-Asp(OtBu)-OH (388 mg, 0.95 mmol, 3 eq) was activated with HATU (358 mg, 0.95 mmol, 3 eq), and DIPEA (328 µL, 1.88 mmol, 6 eq) in DMF (5 mL) at 0 °C for 5 min and then reacted with the resin for 1 h under gentle agitation. After washing the resin with DMF (4 × 10 min × 10 mL) the Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 mL) and the resin washed with DMF (4 × 10 min × 10 mL) before the peptide was extended with Boc-Lys(Fmoc)-OH (445 mg, 0.95 mmol, 3 eq), Fmoc-Lys(N3)-OH (374 mg, 0.95 mmol, 3 eq), Fmoc-Asp(OH)-OtBu (388 mg, 0.95 mmol, 3 eq), Fmoc-Asp(OH)-OtBu (388 mg, 0.95 mmol, 3 eq) and 4,4-bis(4-hydroxyphenyl)valeric acid (162 mg, 0.95 eq, 3 eq) in the indicated order using the same coupling conditions (HATU/DIPEA), Fmoc-deprotection (20% piperidine in DMF) and washing step with DMF mentioned before.

After the last peptide-coupling step, a solution of Cul (18 mg, 0.095 mmol, 0.3 eq), TBTA (16 mg, 0.031 mmol, 0.1 eq) and N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)hex-5-ynamide (261 mg, 0.95 mmol, 3 eq) in a mixture of DMF (2.5 mL) and THF (2.5 mL) was prepared and reacted with the resin at room temperature for 48 hours. After washing with DMF (4 × 10 min × 10 mL), EDTA 50 mM (4 × 10 min × 10 mL) and DCM (4 × 10 min × 10 mL) was washed with DMF (4 × 10 min × 10 mL) and DCM (4 × 10 min × 10 mL).
mL), the compound was cleaved from the resin by agitating with a mixture of TFA (8.6 mL), TIS (1.6 mL), H$_2$O (400 µL), m-Cresol (400 µL) and Thioanisol (400 µL) at room temperature for 1 hour. Cleavage solution was added drop-wise to ice cold diethyl ether (50 mL) obtaining a white precipitate. The pellet was collected by centrifugation, dried under vacuum, redissolved in Millipore water and added with an excess of Tris(2-carboxyethyl)phosphine hydrochloride (30 eq). The product was purified by reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). After lyophilization the final compound was collected as a white powder (91 mg, 70.5 µmol, 22% yield).

HRMS: (m/z) [M+H]$^+$ C$_{52}$H$_{71}$N$_{14}$O$_{19}$S$_3$, 1291.4104, found 1291.4219

**Figure XI.5** Analytical HPLC trace of SM(+)$^{99m}$Tc chelator on a Synergi RP Polar column at a flow rate of 4 mL min$^{-1}$, 5% MeCN in 0.1% aq. TFA to 80% MeCN in 20 min.
2.2.4 Synthesis of SM(+)‐Alexa Fluor594

Compound 4

Chemical Formula: $C_{45}H_{62}N_{12}O_{15}S_2$

Molecular Weight: 1075.18 g/mol

Compound 4 was prepared according to previously described procedures [63].
SM(+)‐Alexa Fluor594

Chemical Formula: $C_{80}H_{94}N_{14}O_{25}S_{4}$
Molecular Weight: 1779.95 g/mol

Amine 4 (0.9 mg, 0.92 µmol, 1.5 eq) was dissolved in degassed TBS (pH 7.6; 1.5 mL). Commercially available Alexa Fluor™ 594 NHS Ester (1 eq., 0.5 mg, 0.61 µmol) was added as a DMF solution (500 µl) and the mixture was stirred at room temperature. After 12 hours, UPLC‐MS indicated completion. The mixture was purified by RP‐HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). Product containing fractions were identified by high‐resolution mass spectrometry, collected and lyophilized overnight to achieve SM(+)‐Alexa Fluor594 as a purple solid (450 µg; 0.25 µmol; 42% yield).

HRMS: ($m/z$) [M+H]$^+$ $C_{80}H_{95}N_{14}O_{25}S_{4}$, 1779.5476, found 1779.6561.
Figure XI.6 Analytical HPLC trace of SM(+)-Alexa Fluor594 on a Synergi RP Polar column at a flow rate of 4 mL min\(^{-1}\), 5% MeCN in 0.1% aq. TFA to 80% MeCN in 20 min.

2.2.5 Synthesis of SM(-)-Alexa Fluor594

![Chemical structure of SM(-)-Alexa Fluor594]

SM(-)-Alexa Fluor594

Chemical Formula: C\(_{98}\)H\(_{120}\)N\(_{18}\)O\(_{32}\)S\(_{3}\)

Molecular Weight: 2158.31 g/mol
Thiol 3 (1.0 mg, 0.78 µmol, 1.5 eq) was dissolved in degassed TBS (pH 7.6; 1.5 mL). Commercially available Alexa Fluor™ 594 C5 Maleimide (1 eq., 0.5 mg, 0.55 µmol) was added as a DMF solution (500 µl) and the mixture was stirred at room temperature. After 12 hours, UPLC-MS indicated completion. The mixture was purified by RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). Product containing fractions were identified by high-resolution mass spectrometry, collected and lyophilized overnight to achieve SM(-)-Alexa Fluor594 as a purple solid (0.33 mg; 0.15 µmol; 28% yield).

HRMS: \( \text{(m/z) } [\text{M+2H}\text{]}^{2+} \text{C}_{98}\text{H}_{122}\text{N}_{18}\text{O}_{32}\text{S}_{3}, \ 1079.3818, \text{found } 1079.4603. \)

**Figure XI.7** Analytical UPLC trace of SM(-)-Alexa Fluor594 on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 mL min⁻¹, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.
3. Statistical analysis of therapy experiment

Differences in tumor volume between therapeutic groups were compared using the two-way ANOVA analysis with Bonferroni post-test of Graphpad Prism 6 (La Jolla, CA, USA).

<table>
<thead>
<tr>
<th>Tumor Size (mm$^3$)</th>
<th>Vehicle vs. $ADC(\cdot)$:</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 16</td>
<td>$p &lt; 0.05$</td>
</tr>
<tr>
<td>day 17</td>
<td>$p &lt; 0.01$</td>
</tr>
<tr>
<td>from day 18</td>
<td>$p &lt; 0.0001$</td>
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<th>Vehicle vs. $ADC(\cdot)$:</th>
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<tr>
<td>day 16</td>
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<td>day 17</td>
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<td>day 18</td>
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<td>from day 19</td>
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<th>Vehicle vs. $SMDC(\cdot)$:</th>
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<tbody>
<tr>
<td>day 17</td>
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<td>day 18</td>
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<td>day 19</td>
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<tr>
<td>day 20</td>
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<td>from day 21</td>
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<table>
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<tr>
<th>Vehicle vs. $SMDC(\cdot)$:</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-significant differences</td>
</tr>
</tbody>
</table>

$ADC(\cdot)$ vs. $ADC(\cdot)$:
| day 25 | $p < 0.0001$ |
| day 26 | $p < 0.0001$ |

$ADC(\cdot)$ vs. $SMDC(\cdot)$:
| day 20 | $p < 0.001$ |
from day 21 \( p < 0.0001 \)

\textit{ADC(+) vs. SMDC(-)}:

day 12 \( p < 0.05 \)
day 13 \( p < 0.01 \)
day 14 \( p < 0.001 \)
from day 15 \( p < 0.0001 \)

\textit{SMDC(+) vs. ADC(-)}:

day 23 \( p < 0.01 \)
day 25 \( p < 0.05 \)
day 26 \( p < 0.01 \)

\textit{SMDC(+)} vs. \textit{SMDC(-)}:

day 13 \( p < 0.05 \)
day 14 \( p < 0.001 \)
from day 15 \( p < 0.0001 \)

\textit{SMDC(-)} vs. \textit{ADC(-)}:

day 13 \( p < 0.05 \)
day 14 \( p < 0.01 \)
from day 15 \( p < 0.0001 \)

\textbf{Body Weight Change (%)}

Vehicle vs. \textit{ADC(+)}:

day 23 \( p < 0.05 \)
day 24 non-significant differences

Vehicle vs. \textit{ADC(-)}:

non-significant differences

Vehicle vs. \textit{SMDC(+)}:

non-significant differences
Vehicle vs. SMDC(-):
non-significant differences

ADC(+) vs. ADC(-):
non-significant differences

ADC(+) vs. SMDC(+):
non-significant differences

ADC(+) vs. SMDC(-):
non-significant differences

SMDC(+) vs. ADC(-):
non-significant differences

SMDC(+) vs. SMDC(-):
non-significant differences

SMDC(-) vs. ADC(-):
non-significant differences
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XI
V.
CURRICULUM VITAE

SAMUELE CAZZAMALLI

Date of birth  December 16th, 1990
Nationality    Italian
Address       Im Maas 3, 8049 Zurich, Switzerland
E-mail        scazzamalli@gmail.com
Telephone     +41 78 67 939 61

EDUCATION

01/2015 – present: PhD studies, ETH Zürich, Department of Chemistry and
Applied Biosciences, Institute of Pharmaceutical Sciences, Switzerland

09/2009 – 09/2014: MSc in Medicinal Chemistry and Pharmaceutical
Technologies (110/110 cum Laude), University of Milan, Department of
Pharmaceutical Sciences, Milan, Italy

Galilei, Crema, Italy

LANGUAGES

Italian native speaker

Fluent English

Scholastic German

PUBLICATIONS

“Enhanced therapeutic activity of non-internalizing Small Molecule-Drug
Conjugates targeting carbonic anhydrase IX in combination with targeted
interleukin-2” Cazzamalli S., Ziffels B., Widmayer F., Murer P., Pellegrini G.,

“Chemically-defined antibody- and small molecule-drug conjugates for in vivo
tumor targeting applications: a comparative analysis” Cazzamalli S., Dal Corso

“Versatile protein recognition by the encoded display of multiple chemical
elements on a macrocyclic scaffold” Li Y., De Luca R., Cazzamalli S., Pretto F.,


ORAL PRESENTATIONS

“Non-internalizing Targeted Cytotoxics for the Treatment of Cancer” Cazzamalli S. and Neri D. ETN Magicbullet Summer School 2017, Como (Italy), 11th – 12th September 2017


“Development of chemoenzymatic continuous flow processes for the preparation of biologically active compounds” Tamborini L., Mastronardi F., Cazzamalli S. and Zambelli P. VIII meeting “Nuove Prospettive in Chimica Farmaceutica (NPCF8)”, Parma (Italy), 9th – 11th June 2014
SELECTED EXTRACURRICULAR ACTIVITIES

• Private tutor in Inorganic and organic Chemistry, Biology and Mathematics (06/09 – 12/14)
• Media Team board member of the Pharmaceutical Scientists' Association (PSA) - ETH Zurich (01/15 – 12/15)
• Social Team board member of the Pharmaceutical Scientists' Association (PSA) - ETH Zurich (01/15 – present)
• Crossfit athlete, Crossfit Kreis 9, Zurich (03/15 – present)