Small molecule-drug conjugates for the treatment of carbonic anhydrase IX expressing solid tumors

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SMALL MOLECULE-DRUG CONJUGATES
FOR THE TREATMENT OF CARBONIC ANHYDRASE IX
EXPRESSING SOLID TUMORS

a thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH
(Dr. sc. ETH Zurich)

presented by

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2014
to my grandfather
Prof. em. Hellmuth Petsche
who inspired my scientific curiosity
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I. SUMMARY

1. English

Systemic chemotherapy with small-molecule cytotoxic drugs remains one of the cornerstones of modern cancer therapy. However, many low molecular weight cytotoxic drugs do not strongly localize to solid tumors but preferentially accumulate in healthy organs. An unfavorable biodistribution, combined with the non-specific mode of action of cytotoxic anticancer agents, can lead to severe side effects, dose limiting toxicities and may prevent the administration of therapeutically effective treatment regimens. Similar considerations may apply to so called “targeted drugs” such as tyrosine kinase inhibitors since many are not as selective as originally believed.

The targeted delivery of highly potent cytotoxic drugs into solid tumors is emerging as promising strategy to overcome the intrinsic limitations of small-molecule anticancer agents. Indeed, some antibodies against accessible tumor markers have the striking ability to selectively accumulate inside neoplastic lesions and have been used for targeted drug delivery applications in the form of antibody drug conjugates.

Yet, antibody carriers may suffer from limitations owing to their high molecular weight and long circulation in plasma. Antibodies may have difficulties penetrating deeply into solid tumors preventing the homogeneous targeting of all tumor cells. Long plasma circulation can lead to premature release of drugs from the antibody due to spontaneous cleavage of the linkage between drug and carrier and lead to unwanted systemic toxicities.

We and others have proposed that small-molecule ligands of tumor cell surface antigens may overcome some of the limitations associated with antibodies as vehicles for targeted drug delivery applications. Small molecules typically penetrate solid tumors more rapidly than large macromolecules and normally have much shorter circulation half-lives.

For the purpose of this study we focused on carbonic anhydrase IX (CAIX) – a tumor cell surface antigen up-regulated in response to hypoxia and strongly expressed in the majority of renal cell carcinoma. Using published structure-activity relationship data and a focused screen against recombinant CAIX, we identified small-molecule ligands that could tolerate the attachment of linkers and payloads yet maintain
binding to their target. Using small animal imaging techniques, we showed that one of our leads, which was derived from the approved anti-glaucoma drug acetazolamide (AAZ), coupled to a near-infrared fluorescent dye, strongly accumulated inside CAIX-expressing SKRC52 renal cell carcinoma xenografts. Using fluorescent microscopy and visible light dyes the remarkable tumor penetration capacity of small molecule conjugates was demonstrated. We furthermore extensively characterized their sub-cellular localization using confocal fluorescence microscopy and flow cytometry. Counter to previous suggestions that CAIX internalized upon ligand binding, we did not see strong internalization in the cell lines studied.

A conjugate consisting of our AAZ-based targeting ligand and the potent cytotoxic maytansinoid DM1, connected through a disulfide linker, was prepared. Intravenous administration of this molecule strongly retarded the growth of SKRC52 xenograft tumors in mice. The therapy was well tolerated with no observable weight loss. Importantly, the standard-of-care drugs sunitinib and sorafenib did not show any benefit in the same model.

Finally, a bivalent AAZ-based ligand was shown to have a higher functional affinity for CAIX in vitro compared to its monovalent counterpart. A bivalent ligand-dye conjugate accumulated inside SKRC52 tumors more strongly at late time points than monovalent controls. In terms of therapeutic efficacy, a proportion of mice bearing SKRC52 xenografts could be sustainably cured using a bivalent ligand-drug conjugate. This had previously not been possible with monovalent ligand-drug conjugates.

To the best of our knowledge, we have developed the first small-molecule based drug delivery strategy targeting CAIX expression in solid tumors. Our conjugates showed remarkable efficacy in a xenograft model of renal cell carcinoma, which did not respond to standard of care drugs sorafenib and sunitinib. Bivalent targeting ligands may be superior delivery vehicles compared to their monovalent counterparts. This is in full analogy to the observation that bivalent antibody formats accumulate more strongly in solid tumors than monovalent ones. We are now further evaluating the therapeutic potential of our drug delivery strategy using different linker-payload combinations and additional models of CAIX-expressing renal cell carcinoma.
2. **Deutsch**


Das hohe Molekulargewicht von Antikörpern bringt jedoch eigene Probleme mit sich. Antikörper haben oft Schwierigkeiten, tief in solide Tumore einzudringen, und können so nicht alle Tumorzellen erreichen. Lange Zirkulationszeiten im Plasma können dazu führen, dass der Wirkstoff spontan frühzeitig vom Antikörper freigesetzt wird, was wiederum zu systemischer Toxizität führen kann.


Unter Verwendung publizierter Struktur-Wirkungs Zusammenhänge und eines fokussierten Screens gegen rekombinante Carboanhydrase IX (CAIX) konnten wir CAIX Liganden identifizieren, die über einen Linker mit Farbstoffen verbunden werden können, dabei aber die Affinität für ihr Target beibehalten. CAIX ist ein Zelloberflächen-Antigen, das in hypoxischen Tumorregionen und konstitutiv in der Mehrzahl von Nieren Kleinzellkarzinomen exprimiert wird. Wir konnten zeigen, dass einer unserer Liganden, der vom zugelassenen Medikament Acetazolamid (AAZ)
abgeleitet ist, verbunden mit einem Infrarot-Fluoreszenzfarbstoff, sich stark in antigen-exprimierenden Xenograft-Tumoren in Mäusen anreichert.


II. INTRODUCTION

In spite of enormous advances in oncology, cancer is still amongst the most prominent causes of death worldwide. In 2012 over 8 million people died of cancer with lung (1.6 million deaths per year) being the most frequent form followed by stomach (720,000 deaths per year), colorectal (690,000 deaths per year) and breast cancer (520,000 deaths per year).[1]

1. Conventional Chemotherapy of Cancer

Besides surgical resection and radiotherapy, chemotherapy is one of the corner stones of cancer treatment. Traditionally, cancer chemotherapy refers to the systemic administration of one or more small molecule cytotoxic agents (i.e., chemical compounds capable of killing cells) with the aim to kill neoplastic cells whilst sparing healthy tissue.[2]

Conventional cytotoxic agents in clinical use typically interfere with cell division. Vinca alkaloids such as vinblastine (1) and taxanes such as paclitaxel (2) inhibit microtubule polymerization and depolymerization, respectively.[3] Both processes are essential for chromosomal separation during cell division. DNA intercalators such as doxorubicin[4] (3) and topoisomerase inhibitors such as irinotecan[5] (4) stall DNA replication. Nucleotide analogues such as 5-fluorouracil (5) inhibit the synthesis of bases required for DNA replication.[6]

Scheme II.1: Clinically approved cytotoxic drugs for the chemotherapy of cancer; structures were obtained from ChEMBL and PubChem databases.
It was originally hoped that chemotherapeutic agents would indeed kill, preferentially, rapidly proliferating cancer cells while sparing normal organs. Many healthy tissues, however, also constantly regenerate (e.g., in the gastrointestinal tract[7], hair follicles[8] or the hematopoietic system[9]). These structures are, consequently, highly susceptible to the action of cytotoxic agents and conventional chemotherapy is typically associated with severe and often dose-limiting side effects.[10-12] DNA alkylating agents such as melphalan (6), which have the potential of damaging non-proliferating cells, can be mutagenic and may lead to secondary malignancies.[13] Researchers are becoming increasingly aware of the fact that many low-molecular weight cytotoxic drugs do not reach tumors with sufficient efficacy and selectivity. As will be discussed in Chapter III in more detail: “Biodistribution studies performed in tumor-bearing mice have shown that the dose of doxorubicin which reaches the neoplastic mass corresponds to only 5-10% of the dose which accumulates in healthy organs (normalizing to organ weight).[14] Similar findings have been reported for other anti-cancer drugs, including $^{18}$F-labeled 5-fluorouracil[15], $^3$H-labeled paclitaxel[16, 17] and Pt$^{II}$ derivatives.[18] Typically, irregular tumor vasculature[19, 20], the expression of drug efflux carriers[21] and high interstitial pressure at the neoplastic site[22] may contribute to the inefficient accumulation of small molecule cytotoxic drugs in solid tumors.

2. **Targeted Cancer Therapies**

Since the early days of chemotherapy enormous efforts have been undertaken to render the pharmacotherapy of cancer more selective and efficacious. Spurred by an ever-expanding understanding of the signaling networks driving cell proliferation[23], protein targets were identified, which were believed to be essential for cancer growth. Many of these represent tyrosine kinases[24] but also the 26S proteasome[25] or the poly ADP ribose polymerase (PARP)[26] have been exploited. It was hoped that selective inhibitors of these proteins (often termed “targeted therapies”) would be highly efficacious and have a more favorable tolerability profile compared to conventional cytotoxic chemotherapy. Indeed, remarkable success has been achieved by the inhibition of the fusion tyrosine kinase BCR-ABL with imatinib (7) for the therapy of chronic myelogenous leukemia (CML).[27]

Resistance against targeted therapies, which have a very specific mode of action, however, often arises.[28, 29] In the case of BCR-ABL, second-generation inhibitors
such as dasatinib (8), nilotinib (9) and bosutinib (10) have been developed to overcome mutations, which render imatinib (7) inefficacious after prolonged administration. Additionally, ponatinib (11), a third generation of BCR-ABL inhibitor, has now been approved for use in patients intolerant or resistant to second-generation inhibitors.  

![Chemical structures](image)

**Scheme II.1:** Clinically approved BCR-ABL inhibitors; structures were obtained from ChEMBL and PubChem databases.

Many tyrosine kinase inhibitors are now known to be less selective than initially believed and may block signal transduction pathways, which are important in healthy tissues. The tyrosine kinase inhibitor sunitinib for example inhibits a large panel of kinases including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) receptor kinases and c-KIT. The side effects, which result from the off-target inhibition of multiple kinases, can thus be considerable. In one study, 79% of patients treated with sunitinib suffered from anemia, 78% from leucopenia, 77% from neutropenia although mostly of grade 1 and 2. Finally, the same limitations in terms of biodistribution as already mentioned for conventional cytotoxic chemotherapies may also apply for targeted therapies. The survivin inhibitor YM155, in its $^{11}$C labeled form, did not visibly accumulate in solid tumors in mice but preferentially localized to the kidneys and liver.

3. **Targeted Delivery of Potent Cytotoxic Drugs**

Targeted drug delivery approaches are promising to improve both the selectivity as well as biodistribution problems associated with conventional and targeted chemotherapy. In these pharmacodelivery strategies, a potent cell poison is
selectively transported into diseased tissue, where the payload is released. If the targeting process is efficient, one would expect that healthy tissues are spared from the action of the cytotoxic agent. Two main approaches for the targeted delivery of highly potent cytotoxic drugs into solid tumors have emerged. Passive targeting approaches (Figure II.1a) make use of the enhanced permeability and retention (EPR) effect. First described by Maeda and co-workers, the EPR effect refers to the observation that macromolecules and nano-sized objects such as albumin, synthetic polymers and nanoparticles often accumulate inside solid tumors. The EPR effect is commonly explained by the fact that tumor vasculature is leaky, nano-sized objects and pass through windows in the epithelium into the tumor tissue and are trapped in the absence of efficient lymphatic drainage. Healthy blood vessels on the other hand are impermeable to the nano-carriers. While nano-carriers were initially prone to accumulate in the liver and spleen, nano-delivery systems such as a polyethylene glycol-polylactic acid (PEG-PLA)/polyethylene glycol-poly(lactic-co-glycolic acid) PEG-PLGA nanoparticle have now been developed, which can have a >10-fold selectivity for the tumor over liver and spleen. Cytotoxic effector molecules encapsulated in the carrier can either be released extracellularly or after uptake into the cancer cell. Alternatively, payloads can be covalently coupled to the surface of the carrier. Linkers can be cleaved extracellularly e.g., by tumor associated enzymes such as matrix metalloproteinases (MMPs) but potentially also intracellularly after uptake of the carrier. Drug encapsulation is typically used for nanoparticulate or liposomal delivery vehicles whilst polymer conjugates typically carry effectors on their surface. In spite of enormous efforts in the field, few nano-drugs have succeeded in the clinic. Pegylated liposomal doxorubicin (PLD, Doxil™) is one of the notable exceptions. Still, in a Phase III trial in breast cancer, PLD was not superior in terms of overall efficacy but only in terms of a reduced cardiotoxicity compared to free doxorubicin suggesting that the biodistribution of the chemotherapeutic agent was affected but tumor uptake did not necessarily increase. Indeed, doubts exist about the generality of the EPR effect and its clinical usefulness. Much like in the case of small molecules, high interstitial pressure and irregular vasculature can oppose EPR-mediated drug delivery.
Figure II.1: Comparison of passive a) and active b) drug delivery approaches a) A nano-carrier (green) loaded with drug (yellow) passes through windows in tumor blood vessels and gets trapped inside the neoplastic tissue. There, the payload can be released by diverse mechanisms. b) A ligand-drug conjugate (green-blue-yellow) extravasates into the neoplastic tissue and binds its target marker (green). Typically, the conjugate gets internalized into malignant cells and the linkage (blue) between ligand (green) and drug (yellow) cleaved intracellularly to release its payload.

Active targeting systems (Figure II.1b) use ligands (e.g., monoclonal antibodies) against accessible tissue-specific markers to deliver payloads into the desired tissue.\cite{38, 49, 54} In the context of cancer therapy, ligands against tumor markers are used to deliver payloads into neoplastic lesions. Typically one or more payload molecules are covalently attached to the targeting ligand through cleavable linkers. These constructs are hereafter referred to as ligand-drug conjugates. After administration to the patient, the ligand-drug conjugate initially circulates in the blood stream but eventually extravasates (i.e., leaves the blood vessels). In tissue expressing the target marker, the conjugate binds and gets trapped. In marker-negative tissue, binding does not occur and the conjugate is eventually washed out again.

To release the drug, it has previously been postulated that target marker-conjugate complexes must internalize into target cells. The changed intracellular environment (e.g., reducing agents in the endosome, a lowered pH and proteases in the lysosome) would then trigger linker cleavage and release the payload.\cite{54, 55} Our group, however, has recently shown that antibody-drug conjugates (ADCs) with binding specificity against the angiogenesis associated ectodomain A of fibronectin (EDA), an extracellular matrix component, could release sufficient drug extracellularly.
to elicit cures in at least one tumor model.\textsuperscript{56, 57} In this case, a disulfide bridge was used as the cleavable linkages between the drug and the antibody.

4. **Aims and Structure of this Thesis**

It was the aim of the research presented in this cumulative thesis to develop a novel actively targeted drug delivery platform, using small molecule ligands of the cell-surface tumor marker carbonic anhydrase IX (CAIX)\textsuperscript{58} as the targeting vehicle. Until recently, antibodies against accessible cell surface markers of disease have mainly been used for targeted drug delivery applications.\textsuperscript{49, 54} We and others, however, believe that small molecules may have significant advantages over conventional antibodies for drug delivery applications.\textsuperscript{38}

The rationale of this thinking, recent examples of small-molecule targeted drug delivery systems as well as technologies for the discovery of small targeting ligands are discussed in Chapter III, which corresponds to our review “Small targeted cytotoxics: current state and promises from DNA-encoded chemical libraries.” published in Angewandte Chemie in 2013.\textsuperscript{38} In that section, tumor cell surface antigens as potential targets, linker systems and cytotoxic payloads are also presented. The chapter is intended to serve as a general introduction to the field of small molecule drug conjugates.

Chapter IV briefly introduces CAIX as a tumor cell surface antigen and therapeutic target in particular in the context of renal cell carcinoma.

Chapter V corresponds to our paper “A small-molecule drug conjugate for the treatment of carbonic anhydrase IX expressing solid tumors” published in Angewandte Chemie in 2014. The chapter describes the evaluation of CAIX ligands for targeted drug delivery applications as well as the first example of a successful therapy of CAIX-positive renal cell carcinoma using a small molecule-drug conjugate.\textsuperscript{59}

Chapter VI corresponds to the paper “A bivalent small molecule-drug conjugate directed against carbonic anhydrase IX can elicit complete tumour regression in mice” published in Chemical Science in 2014. It describes our attempt to improve the targeting performance of our ligand by dimerization.\textsuperscript{60} The impact of bivalency on therapeutic potency is discussed.

Chapter VII is a critical discussion of the presented work going beyond the discussion already presented in Chapters V and VI. It contains personal considerations and an
outlook for the next steps in our research program and its potential for clinical development.

Chapters VIII and IX correspond to the material and methods sections of our primary research papers reproduced in Chapters V and VI and are followed by references, acknowledgements and my curriculum vitae.
III. SMALL TARGETED CYTOTOXICS

This chapter corresponds to the review “Small targeted cytotoxics: current state and promises from DNA-encoded chemical libraries” by N. Krall, J. Scheuermann, D. Neri first published in Angewandte Chemie International Edition 2013, 52, 1384-402. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission. Minor additions were made to section 2.2. to reflect recently released results from clinical trials with folate-drug conjugates.

1. Introduction

The use of cytotoxic agents (i.e., chemical compounds capable of killing cells) is a core component of pharmacological approaches for the therapy of cancer and of other serious pathologic conditions (e.g., rheumatoid arthritis\textsuperscript{[61]}, lupus\textsuperscript{[62]}, multiple sclerosis\textsuperscript{[63]}). Ideally, cytotoxic agents should preferentially kill disease-associated cells (e.g., cancer cells), while sparing healthy tissue. In practice, the situation is much more complex.

Many anticancer drugs inhibit cells in rapid proliferation\textsuperscript{[2]} and, thus, have an impact on normal adult tissues, which constantly regenerate (such as epithelial structures in the gastro-intestinal tract and in the skin, hair growth, haematopoiesis). However, a more fundamental problem associated with the use of low-molecular weight compounds for therapeutic applications relates to the ability of small molecules to localize at sites of disease \textit{in vivo}. Indeed, many low-molecular weight cytotoxic drugs do not preferentially accumulate in solid tumors. Biodistribution studies performed in tumor-bearing mice have shown that the dose of doxorubicin which reaches the neoplastic mass corresponds to only 5-10% of the dose which accumulates in healthy organs (normalizing to organ weight).\textsuperscript{[14]} Similar findings have been reported for other anti-cancer drugs, including \textsuperscript{18}F-labeled 5-fluorouracil\textsuperscript{[15]}, \textsuperscript{3}H-labeled paclitaxel\textsuperscript{[16, 17]} and Pt\textsuperscript{II} derivatives (Figures III.1a and b).\textsuperscript{[18]}

The accumulation of small organic drugs in solid tumor masses is hindered by the high interstitial pressure at the tumor site\textsuperscript{[64]}, irregular vasculature\textsuperscript{[19, 20]} and by the fact that tumor cells frequently over-express multidrug resistance proteins.\textsuperscript{[21]}
Consequently, there is a considerable interest in the development of targeted cytotoxic agents, capable of selective localization at the site of disease, which may spare healthy tissue and help overcome the intrinsic limitations of conventional cytotoxic drugs.

![Figure III.1](image)

**Figure III.1:** a) Tissue distribution of $^{3}$H-labeled paclitaxel ($^{3}$H-PTX) in tumor-bearing mice.\[^{17}\] b) Tissue distribution of doxorubicin (DOX) in tumor-bearing mice.\[^{14}\] c) Macroscopic accumulation of an antibody against a splice isoform of tenasin C in a solid tumor. d) Trapping of fluorescently labeled trastuzumab (green) outside tumor blood vessels (red; reproduced by permission from the American Association for Cancer Research\[^{65}\]).

1.1. Antibodies as Vehicles for the Targeted Delivery of Cytotoxic Drugs

Antibodies have been investigated for many years as attractive vehicles for the targeted delivery of diverse payloads.\[^{66, 67}\] Nowadays, human monoclonal antibodies can be raised against virtually any target protein of interest\[^{68}\] and certain antibodies have exhibited a striking ability to selectively localize in solid tumors (Figure III.1c)\[^{69-73}\] and at other sites of disease, such as in rheumatoid arthritis\[^{74}\], endometriosis\[^{75}\] and atherosclerosis.\[^{76}\]

Since cytotoxic drugs usually display their action inside the cell, it has long been postulated that antibodies, capable of selective internalization into the target cell and equipped with cleavable linkers, would be ideally suited for the targeted delivery of cytotoxic agents.\[^{66, 67}\] Recently, it has, however, become clear that antibody internalization is not a strict requirement for targeted drug delivery, as antibody-drug
conjugates specific to extracellular matrix proteins can mediate a potent anti-tumor effect if equipped with disulfide-based cleavable linkers.\cite{57, 77}

While it remains open which classes of target antigens and which chemical coupling strategies may be preferable for the development of therapeutic antibody-drug conjugates, research efforts in this field have led to the recent approval of Adcetris, a conjugate of the CD30 specific antibody brentuximab and monomethyl auristatin connected through a protease-cleavable linker\cite{78-80}, for the treatment of Hodgkin lymphoma. Furthermore, more than 30 antibody-drug conjugates are currently in clinical testing, primarily for applications in oncology.

The concept of using antibody-drug conjugates for the targeted delivery of cytotoxic agents to each tumor cell within a neoplastic mass is seductively simple but the \textit{in vivo} implementation of the technology can be much more difficult. There are a number of limitations for the performance of antibody-drug conjugates, which mainly relate to their ability to reach cells within the tumor mass. Furthermore, the cost-of-goods for antibody-drug conjugates can be substantial, reflecting the need for the separate industrial manufacturing of clinical-grade antibody by fermentation technology, of clinical-grade drug and of the resulting conjugate in large quantities.

The limitations of antibodies as targeting vehicles, which are discussed in more detail in Section 1.2, provide a strong motivation to explore alternative strategies for the targeted delivery of cytotoxic drugs, which may reach diseased cells more efficiently \textit{in vivo} and which may be easier to produce (\textit{e.g.}, by chemical synthesis).

1.2. Limitations of Antibodies as Delivery-Vehicles for Cytotoxic Drugs

Tumors are not homogeneous masses of identical cells and loss of antigen expression is not infrequent in cancer.\cite{81} Furthermore, the “antigen barrier” hypothesis has been postulated as a main obstacle to a homogeneous antibody penetration into the tumor mass.\cite{82-84} Indeed, large macromolecules (including antibodies) do not efficiently extravasate (\textit{i.e.}, get out of blood vessels) and diffuse into tissue. The few antibody molecules, which reach the abluminal side of blood vessels, are immediately trapped by antigen located on perivascular tumor cells, preventing the targeting of tumor cells distant from blood vessels (Figure III.1d).\cite{65}

The tumor-targeting performance of many monoclonal antibodies has been extensively studied in tumor-bearing mice using radiolabeled protein preparations
and quantitative biodistribution analysis. However, similar studies (e.g., by nuclear medicine imaging techniques or by autoradiographic analysis of biopsies) are typically not performed in patients during industrial drug development programs, with some notable exceptions.\cite{85-88} Seduced by the preferential antibody accumulation at site of disease at late time points following intravenous administration, it is easy to forget that the vast majority of injected antibody molecules do not reach their target \textit{in vivo}, while virtually all of them accumulate (at least transiently) in excretory organs (liver for intact antibodies, kidneys for small antibody fragments).\cite{89} Indeed, variations in off-target toxicity associated with clearance may make the crucial difference for the development and registration of a cytotoxic agent (e.g., oxaliplatin).\cite{90}

The use of smaller antibody formats does not substantially overcome the pharmacokinetic problems associated with the use of macromolecules as targeting agents. Most antibody formats extravasate slowly. Intact immunoglobulins can display efficient tumor targeting, but they do so at the expense of long circulatory half-lives.\cite{72, 73, 89} To the other extreme, small antibody formats (such as scFv fragments) are cleared more rapidly from circulation but also exhibit reduced tumor uptake.\cite{89, 91}

Finally, antibodies can be immunogenic, even when they are humanized or fully human.\cite{92} The development of an undesired antibody reaction may not only cause hypersensitivity in patients, but also neutralize the therapeutic effect of antibody drugs and alter pharmacokinetic properties.

1.3. Tissue Distribution and Properties of Low-Molecular Weight Compounds

As mentioned in Section 1.1, small organic drugs typically do not preferentially accumulate in solid tumors, mainly because there is nothing that preferentially keeps them there. However, in contrast to antibodies, small molecules can diffuse out of blood vessels in a matter of seconds. Small molecules can also rapidly penetrate deep into tissues, as one can visualize by Hoechst 33342 nuclear staining at different time points after intravenous administration.\cite{93}

The potential benefits associated with the use of small tumor targeting agents are best exemplified by the analysis of “pre-targeting” strategies, where bispecific antibodies (capable of recognizing a tumor-associated antigen and a metal chelator)
are allowed to localize at tumors and clear from circulation, before being targeted by a low molecular weight radiometal-chelator complex.\cite{94, 95}

A direct comparison of tumor uptake and tissue distribution values for radiolabeled antibodies specific to a tumor-associated antigen (e.g., carbonic anhydrase IX) and for the corresponding pre-targeting implementation (Figure III.2a and b) of the same antibodies showed that the radiometal-chelator complex exhibited percent injected dose per gram % ID g\(^{-1}\)) values in the tumor which were at least comparable to those of the corresponding antibodies. However, this efficient tumor uptake was achieved much more rapidly in the case of the pre-targeting approach, with strikingly good tumor to organ ratios (>10:1) already one hour after injection.\cite{94}

![Figure III.2: a) Direct delivery of a payload (yellow) with an armed antibody specific for a marker of disease (green). b) Pretargeting with a bispecific antibody binding a marker of disease (green) and a payload (yellow) administered after clearance of the antibody from healthy tissue. c) Direct delivery of a payload (yellow) using a small ligand against a marker of disease (green).](image)

Taken together, these considerations suggest that a novel class of targeted cytotoxic agents, consisting of a low molecular weight ligand with disease homing properties coupled to a cytotoxic drug with a suitable linker, hereafter often called “small molecule-drug conjugates”, may overcome some of the limitations of antibody-drug conjugates. Indeed, small ligand-targeted drugs (Figure III.2c) are expected to rapidly reach their target \textit{in vivo}, to be non-immunogenic and to be amenable to chemical synthesis.

2. \textbf{Natural Small Delivery Vehicles and Their Analogs}

The search for smaller vehicles with potentially favorable pharmacokinetic properties for the targeted delivery of cytotoxic agents has so far mainly focused on naturally occurring molecules, such as small peptides\cite{96-99} and vitamins.\cite{100, 101} These ligands often display high affinities to their cognate receptors and undergo a rapid receptor-mediated endocytosis after binding. Alternatively, substrate analogs have been
investigated as potent binders for tumor associated enzymes, such as prostate specific membrane antigen.\textsuperscript{[102, 103]}

Upon ligand binding to the target cell, the ligand-receptor complex folds into a vesicle, which subsequently fuses with the early endosome. A payload may be released at this stage by disulfide cleavage or the lowered pH. Alternatively, the targeted cytotoxic agent may proceed to the lysosome, where drug release may occur through protease and esterase activities or through a further pH reduction. Finally, the drug needs to escape the endosome or lysosome by passive diffusion, in order to act on cytosolic or nuclear targets (Figure III.3).\textsuperscript{[104, 105]}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Mechanism of uptake and activation of ligand-targeted drug conjugates.}
\end{figure}

\section{2.1. Small Peptide Based Delivery Systems}

A large number of peptide receptors are highly overexpressed in various types of cancer. Their ligands, which are typically much smaller than antibodies (usually 10 to 30 amino acids long) and hydrophilic, have been proposed for the targeted delivery of radionuclides or cytotoxic agents to solid tumors.\textsuperscript{[106, 107]}

Since natural peptides often suffer from short plasma half-lives or very narrow receptor subtype specificities, derivatives with improved properties have been developed often by the introduction of unnatural amino acids.\textsuperscript{[106]} Several radiolabeled peptides have now reached the clinic showing very encouraging performance as imaging or radiotherapeutic agents, providing a validation for the peptide-targeting approach.\textsuperscript{[108, 109]}
Pioneering work in the area of peptide-drug conjugates from the group of Andrew Schally revealed that derivatives of luteinizing hormone-releasing hormone (LH-RH) could be used for the delivery of the cytotoxic agents cisplatin and [trans-bis(salicylaldoximato)copper(II)]. Subsequently, conjugates of somatostatin, bombesin and LH-RH analogs as the targeting ligands and doxorubicin or 2-pyrrolinodoxorubicin as the active drug were prepared. Usually, a Lys side chain or the terminal amine of the peptide and the primary alcohol of the drug were connected through a glutaryl spacer (Scheme III.1). In vitro, constructs typically exhibited good receptor-binding affinities (i.e., \(K_D\) values in the low single digit nanomolar range as estimated from competition experiments against radiolabelled peptides using receptor positive whole cells or membrane preparations), yet cytotoxicity was found not to be increased compared to the unmodified drug. In vivo, such conjugates exhibited more potent tumor growth-retardation than the cytotoxic payload alone with lower accompanying toxicities in several models of cancer. Importantly, the peptide alone did not have any effect on tumor growth and unmodified peptide was able to suppress the therapeutic action of targeted peptide-drug conjugates. In spite of the potentially stable nature of the linker no evidence was provided about the efficiency, mechanism and site of drug release. Nonetheless, AN-152 1, a LH-RH derivative conjugated to doxorubicin glutaryl ester, has recently completed Phase I clinical studies for endometrial, ovarian, prostate and bladder cancer.

Scheme III.1: The peptide-targeted drug-conjugate AN-152 1 consisting of [D-Lys6] luteinizing hormone-releasing hormone (LH-RH) as the targeting moiety and doxorubicin (DOX) as the cytotoxic payload linked through a glutaryl spacer.
Related peptide ligands have also been tested \textit{in vivo} as conjugates with other drugs. For example, Kompella and coworkers reported a conjugate of docetaxel glutaryl ester linked to a Ser residue of deslorelin, a very potent LH-RH agonist\textsuperscript{122}. The conjugate exerted an up to 15-fold increased antiproliferative effect on LNCaP and PC-3 human prostate cancer cells ($IC_{50} = 0.29 \pm 0.03$ nM and $0.42 \pm 0.06$ nM respectively) over docetaxel ($IC_{50} = 4.47 \pm 0.93$ and $6.26 \pm 1.24$ nM respectively) \textit{in vitro} and also showed improved tumor growth retardation in PC-3 xenografts \textit{in vivo}. Coy and coworkers coupled the cytotoxic compound camptothecin to analogs of somatostatin and bombesin through carbamate and $N$-methyl carbamate linkers\textsuperscript{123, 124}. In the case of somatostatin, the resulting conjugates were generally less potent antiproliferative agents than free camptothecin when tested against a range of human cancer cell lines \textit{in vitro} ($IC_{50} = 64.1$ nM - $4.83$ $\mu$M for the conjugates compared to $3.10 - 259$ nM for the free drug), possibly because of the high linker stability. Some growth retardation of NCI-H69 human small cell lung cancer xenografts in nude mice was nevertheless observed. CA20948 rat pancreatic tumor cells on the other hand were more sensitive to the conjugates ($IC_{50} = 1.36 - 1.79$ $\mu$M) than to free camptothecin ($IC_{50} = 3.08$ $\mu$M) \textit{in vitro} and tumor growth was also slowed down \textit{in vivo}. In the case of bombesin, the conjugate CPT-L2-BA3 exhibited varying cytotoxicities against a range of human cancer cell lines ($IC_{50} = 33$ nM - $2.7$ $\mu$M) and some tumor growth retardation in NCI-H1299 xenograft bearing mice.

In summary, conjugates of potent cytotoxic drugs with peptide ligands of cancer-associated receptors have shown promising results in several experimental models of cancer. Drugs linked to the carrier vehicle through more labile ester linkages are often more active than conjugates with more stable amide and carbamate connectors. However, systematic studies addressing the mechanism of drug release, as well as tumor-homing potential of the conjugate by quantitative biodistribution studies, are still missing.

### 2.2. Vitamin Based Drug Delivery Systems

Many tumors have an increased requirement for vitamins essential for biosynthesis and nutrient metabolism such as folate (2), biotin (3) and cobalamin (4) (Scheme III.2) due to their very rapid cell division cycles\textsuperscript{125}. The corresponding cell surface
uptake-transporter proteins are thus often overexpressed on tumor cells and have been exploited for the targeted delivery of various therapeutic effectors.\cite{100, 104, 105, 126}

**Scheme III.2:** Vitamins commonly used for drug delivery applications. The functional groups on folate (2) and biotin (3) amenable to payload attachment are shaded in blue.\cite{100, 101, 105} Payloads attached to the yellow shaded groups on cobalamin (4) have been shown to maintain full binding to all cobalamin trafficking proteins whilst payload attachment through the group shaded in green allows the generation of transcobalamin I selective constructs.\cite{126}

The most extensively studied example of vitamin based drug delivery is the folate (2) / folate receptor (FR) system. Folate conjugates typically retain high binding affinities for the FR ($K_D = 1-10$ nM\cite{100, 127}) which is abundantly over-expressed in many malignant tumors (e.g., up to 80 pmol (mg membrane protein)$^{-1}$ in KB cells\cite{128}) and can rapidly shuttle conjugates into the cell (e.g., at a rate of up to $3.4 \times 10^5$ molecules cell$^{-1}$ h$^{-1}$ in KB cells\cite{129}). Radiolabeled analogs (e.g., 5) and various fluorophores (e.g., 6) have been used as folate-conjugates in imaging studies (Scheme III.3).\cite{130-136} Folate tethered protein toxin pseudomonas exotoxin (PE38, cytotoxicity down to $IC_{50} = 20$ pM for a disulfide bridged conjugate in FDHeLa cells)\cite{137} and conjugates with the natural product cytotoxics camptothecin ($IC_{50} = 10$ nM in KB cells)\cite{138}, desacetylvinblastine ($IC_{50} = 11$ nM in KB cells)\cite{139-141}, the maytansinoid DM1 ($IC_{50} = 16$ nM in KB cells)\cite{128}, tubulysin B ($IC_{50} = 7$ nM in KB cells)\cite{142, 143} and mitomycin C ($IC_{50} = 5$ nM in KB cells)\cite{144, 145} have been investigated as targeted therapeutics. Folate-coated liposomal drug carriers have been reported\cite{146-148} as well as hapten based immunotherapy\cite{149} by which the immune system of a host organism previously sensitized against fluorescein was directed to attack tumor cells decorated with folate-fluorescein conjugates. Dual drug conjugates consisting of a folate homing ligand, with mitomycin C and vinblastine as payloads, have recently been
proposed.\textsuperscript{150} As a result of these research efforts, three different folate-drug conjugates and one imaging agent are currently being investigated in clinical trials.\textsuperscript{151, 152} In particular, EC145 (7) ($IC_{50} = 11$ nM in KB cells) had reached Phase III testing in patients with ovarian cancer (Scheme III.3). This class of molecules has recently been covered in a number of excellent reviews.\textsuperscript{100, 105, 153} For this reason, we will summarize here only the findings, which are most relevant to our discussion.

Scheme III.3: $^{99m}$Tc labeled folate-conjugate EC20 (5)\textsuperscript{136} and disulfide FRET probe 6\textsuperscript{127} for imaging applications, vinblastin-folate conjugate EC145 (7)\textsuperscript{140, 141} for targeted drug-delivery to folate-receptor positive tumors.

Folate-based radiopharmaceuticals rapidly accumulate at the tumor site and quickly clear from FR-negative tissue.\textsuperscript{111} Indium-diethylenetriamine pentaacitic acid (DTPA)-folate, previously shown to be taken up in a FR-dependent manner \textit{in vitro}, attained maximum tumor accumulation already 30 minutes after injection in xenograft mouse models.\textsuperscript{133} Absolute tumor uptake values were good (up to 10% ID g$^{-1}$) and essentially stable over a period of 24h. Efficient clearance from FR-negative tissue occurred in less than 1h with the exception of kidneys and bladder, which remained radioactive for prolonged periods of time. It should, however, be kept in mind that kidneys, in addition to representing a major route for the excretion of small organic
molecules, also express the folate receptor, potentially leading to unwanted active conjugate uptake.\cite{154} It is not known whether also the radiolabeling methodology influenced kidney uptake values, but similar results were reported for $^{99m}$Tc-labeled radio-folates.\cite{131,134}

The use of fluorescently-labeled folate analogs was crucial for the demonstration of a rapid extravasation of this class of small organic tumor targeting agents. Folate-rhodamine conjugates were studied in vivo using multi-photon microscopy, revealing that these agents rapidly exit blood vessels and that tumor folate receptors could be saturated within 5 minutes after intravenous administration. Periorbital and intraperitoneal administration gave rise to $< 30$ min and $< 100$ min saturation times. In the same study it was also shown that a “binding site barrier” (see also Section 1.3) was present but its overall effect on tumor uptake was negligible at saturating doses ($300$ nmol kg$^{-1}$).\cite{155} These results make a strong case for the use of small organic ligands if rapid clearance and rapid accumulation at site of disease are needed.

The discovery that activated macrophages could take up folate in an FR dependent manner\cite{156} opened the possibility to image sites of inflammation\cite{157} and, potentially, to devise pharmacodelivery intervention schemes for diseases such as arthritis, psoriasis, Crohn’s disease, atherosclerosis and others where activated macrophages play a pivotal role.\cite{158} Similar findings have previously been reported for the uptake of biotin at sites of infection.\cite{159}

As a first attempt, fluorescein hapten-based immunotherapy showed promising results in the treatment of animal models of arthritis.\cite{160} Recently, a folate-aminopterin conjugate has been reported which shows high activity against the adjuvant induced arthritis model in rats.\cite{161} A didemnin B-folate conjugate has been shown to exhibit potent ($IC_{50} = 13$ nM) cytotoxicity and anti TNF-\alpha activity in RAW264.7 macrophages.\cite{162}

Initial evidence for the importance of the chemical nature of the linker connecting folate and its payloads came from studies with folate-pseudomonas exotoxin (PE38) conjugates.\cite{137} Using a reducibly cleavable disulfide bond as the linkage, observed cytotoxicity was 4 orders of magnitude above that of a conjugate based on a stable thioether bond. The release of payloads from folate-based conjugates by disulfide cleavage was further investigated directly using the folate Förster Resonance Energy Transfer (FRET) probe $6$.\cite{127} It was demonstrated that the probe never reached the lysosome. In fact, disulfide cleavage was already observed in the endosome from
which the folate-FR complex seemed to be directly recycled to the cell surface. From fluorescence based pH measurements in endosomal compartments it was concluded that the environment was not sufficiently acidic to cleave acid labile drug-folate linkages.\textsuperscript{[163]} The comparison of two folate-conjugates to desacetylvinblastine monohydrazide conjugated to folate either through a self-immolative disulfide based linker or an acid labile hydrazone linkage in vivo further underpinned these findings. Whilst the disulfide based conjugate showed remarkable activity, the hydrazone based conjugate only exhibited moderate tumor growth retardation.\textsuperscript{[140]} Similarly, a taxol-folate conjugate based on an acid labile ester linkage has been shown to have lower therapeutic efficacy than the free taxol drug alone.\textsuperscript{[164]}

The most comprehensive clinical dataset is currently available for EC145 (7). In a phase II clinical study it was tested in combination with pegylated liposomal doxorubicin (PLD) against PLD alone in patients with platinum resistant ovarian cancer (PRECEDENT trial, www.clinicaltrials.gov). Median progression free survival (PFS) was 5.0 months in the combination group and 2.7 months in the PLD-only group. Grade 3 and 4 adverse events were statistically significantly higher in the combination group (76%) compared to the monotherapy (54%, \( p = 0.009 \), Fisher’s exact test). Pre-screening of ovarian cancer patients with \( ^{99}\text{Tc} \)-based imaging agent EC20 (5) revealed a markedly improved overall survival in patients only carrying FR-positive lesions (FR(100%) group, 14.6 months) compared to patients with FR-negative disease (FR(0%) group, months).\textsuperscript{[165]} The patient population was, however, small (43 individuals) and only 7% of patients of the FR(100%) group experienced a complete or partial response. A phase III study (PROCEED trial, www.clinicaltrials.gov) was initiated but suspended. Clinical trials with other folate-drug and folate-hapten conjugates are now ongoing (www.clinicaltrials.gov).

Besides folate, also cobalamine (vitamin B\textsubscript{12}, (4)), which is essential for thymidine biosynthesis, has actively been investigated as a ligand for the delivery of cytotoxic drugs into solid tumors.\textsuperscript{[126]} Indeed, \( ^{57}\text{Co-Vitamin \text{B}_{12}} \) was investigated for imaging applications in murine models of cancer. The translation of this approach into the clinic was hampered by the high uptake in the liver, pancreas and kidneys.\textsuperscript{[166]}

Nevertheless, cobalamin conjugates with chelators for radiometals, which are more suitable for imaging in humans such as \( ^{99}\text{Tc} \) and \( ^{111}\text{In} \), were prepared\textsuperscript{[167, 168]} as well as several conjugates to derivatives of cisplatin\textsuperscript{[169]} and an acid cleavable conjugate to the microtubule stabilizing agent colchicine.\textsuperscript{[170]} In all of these approaches the
therapeutic payloads were attached at modification sites which were known to allow constructs retain binding affinity for all physiologically important cobalamin carriers namely transcobalamin I (TCI, haptocorrin), intrinsic factor (IF) and transcobalamine II (TCII). Only later it was realized that whilst TCII and the corresponding TCII receptor were responsible for uptake of cobalamin from the bloodstream into normal tissues, membrane bound TCI was highly expressed in certain types of tumors. Cobalamine conjugates which did not bind TCII but retained binding affinity for TCI were thus synthesized and showed improved targeting properties in vivo.[171]

Similar to the situation encountered with folate and vitamin B₁₂, it has recently been shown that biotin receptors are overexpressed in many cancer cells.[125] The group of Iwao Ojima described several conjugates of biotin (3) to fluorescein, coumarin, a taxoid-fluorescein derivative and the improved taxoid SB-T-1214.[101] The authors were able to show that L1210FR leukemia cells could efficiently take up biotin-fluorescein conjugates. Furthermore, a self-immolative disulfide linker could be cleaved intracellularly as demonstrated by activation of quenched coumarin fluorescence. The biotin-linker-taxoid-fluorescein conjugate was also efficiently cleaved resulting in the green fluorescence labeling of microtubule bundles. Finally, whilst the free taxoid exhibited low nanomolar cytotoxicity (IC₅₀ = 9.5 – 10.7 nM) against biotin positive and negative cell lines, the targeted cytotoxic selectively killed biotin receptor positive cells with a potency equal to the free taxoid (IC₅₀ = 8.8 nM).

2.3. Substrate Analogs
Prostate specific membrane antigen (PSMA, also known as glutamate carboxypeptidase II) is a well-established marker of prostate cancer and also occurs in the neo-vasculature of many solid tumors.[172, 173] In one study, tumor cell surface PSMA expression levels of 292 – 4,196 ng (mg protein)⁻¹ were detected in a set of 5 human prostate cancer samples but similar levels were found in normal prostate tissue.[174] Analogs of N-acetylaspartyl glutamate (i.e., the natural substrate of PSMA, Scheme III.4) 8-11 are potent inhibitors of the enzyme and have been used as homing ligands for tumor imaging[175, 176], targeted delivery of cytotoxics[102, 103, 177, 178] and also hapten based immunotherapy.[179]

The first PSMA-ligand drug conjugate consisting of a urea based PSMA inhibitor, a glutaryl linker and doxorubicin, was described by Alan Kozikowski and coworkers.[103]
The product efficiently blocked the binding of a radioactive competitor to recombinant PSMA dimer. *In vitro* cytotoxicity, however, was markedly lower compared to free doxorubicin (at 5 µM only 30% C4-2 proliferation was inhibited) possibly due to the stable nature of the amide linker.

Very potent PSMA based targeted cytotoxics have since then been reported by the group of Philip Low.\[102, 177\] Using 2-[3-(1,3-dicarboxypropyl)-ureido]pentanedioic acid (DUPA, *K*<sub>i</sub> = 8 nM against the purified enzyme\[102\]) 11 as the targeting ligand, a conjugate with fluorescein isothiocyanate (FITC) was shown to bind to LNCaP cells and could be outcompeted by a different PSMA inhibitor. Corresponding results were obtained with DUPA-Rhodamine B in uptake studies.\[102\] DUPA-\textsuperscript{99m}Tc was investigated as a radio-imaging agent (*K*<sub>D</sub> = 14 nM towards LNCaP cells *in vitro*).\[102\] High tumor uptake (11.2% ID g<sup>-1</sup>) was observed alongside with kidneys (28.9%), which, however, can at least partly be explained by the expression of PSMA in murine kidneys. Finally, a conjugate of tubulysin B hydrazide connected to DUPA via a self-immolative disulfide linker 12 showed potent inhibition of [*3*H]-thymidine incorporation (*IC*<sub>50</sub> = 3 nM) in LNCaP cells *in vitro* and sustained tumor regression in LNCaP xenografts with no observable toxicity *in vivo*.\[102\] DUPA drug conjugates with vinblastine, camptotecin, verrucarin A and didemnin B have been investigated and exhibited potent activity with exact cytotoxicity *IC*<sub>50</sub> values depending on the nature of the drug.\[177\] An approach related to targeted delivery described by Peter Schultz and co-workers merits mentioning here: The PSMA ligand DUPA (11) was stably linked to a highly immunogenic 2,4-dinitrophenyl (DNP) group. The conjugate was injected into LNCaP bearing SCID mice followed by anti-DNP serum raised in immune competent mice and showed significant NK cell dependent antitumor activity.\[179\]
Scheme III.4: a) PSMA-catalysed cleavage of N-acetylaspartyl glutamate (NAAG) to N-acetylaspartate and glutamate\textsuperscript{[175]} b) NAAG-analog inhibitors of PSMA \textsuperscript{8-11} capable of delivering payloads into solid tumors\textsuperscript{[102, 103, 175-179]} Groups, which tolerate attachment of a payload, are marked in blue. c) A conjugate of the PSMA-inhibitor DUPA (\textsuperscript{11}) with tubulysin B connected through a peptide spacer and self-immolative disulfide linker \textsuperscript{12}\textsuperscript{[102, 177]}

3. Validated Targets for Pharmacodelivery

Traditionally, ligand-based pharmacodelivery strategies have relied on specific binders (most often, monoclonal antibodies) to accessible antigens selectively expressed by diseased cells\textsuperscript{[66]}. In the case of tumor targeting applications, one would typically raise antibodies to the extracellular portion of a membrane protein, which is over-expressed at the tumor site relative to normal tissue. More recently, components of the modified extracellular matrix at the site of disease\textsuperscript{[180, 181]}, as well
as markers of angiogenesis located at newly formed blood vessels\cite{182, 183} have been considered as alternative targets for pharmacodelivery applications. The ligand-based targeting of tumor cells is hindered by the possible loss of antigen expression\cite{81} and by the difficulty of reaching cancer cells, which are far away from the nearest tumor blood vessels.\cite{65} By contrast, markers expressed in the tumor neovasculature and in the sub-endothelial extracellular matrix may be more accessible, abundant and genetically stable, allowing an efficient tumor targeting with long residence time for the ligand at site of disease.\cite{70}

In the cancer field, a number of tumor-associated enzymes with druggable pockets have been described and validated for pharmacodelivery applications using monoclonal antibodies. They include carbonic anhydrase IX (a marker of hypoxia, which is over-expressed in the majority of kidney cancer cells as a result of von Hippel-Lindau mutations)\cite{58, 184}, placental alkaline phosphatase (an excellent marker of ovarian cancer)\cite{185}, matrix metalloproteinase-3\cite{186, 187} and PSMA in prostate cancer\cite{172, 173}, to name just a few. Validated receptor tyrosine kinases, such as EGFR and HER2/neu\cite{188}, have their catalytic domain inside the cell and are thus less amenable to being targeted with enzyme inhibitors for drug delivery purposes. However, peptidomimetics with dissociation constants for HER2/neu in the submicromolar range have been reported.\cite{189} The identification of small organic ligands specific to proteins involved in protein-protein interactions (e.g., extracellular matrix proteins) is considerably more difficult and requires innovative approaches to chemical library design and screening.\cite{190}

It is worth mentioning that, in principle, the selective expression of certain enzymatic activities at site of disease (e.g., hydrolytic enzymes) could be used for the tissue-specific conversion of inactive prodrugs into the corresponding cytotoxic agent. These strategies are extremely elegant and possess, in our opinion, a high pharmaceutical potential for the development of selective drugs. However, this field is outside the scope of this review, but has been covered in the recent past.\cite{191}
4. Discovery of Homing Ligands with DNA Encoded Libraries

The ability to identify lower molecular-weight ligands specific to accessible markers of pathology (e.g., tumor-associated antigens) is crucial for the development of a novel class of smaller targeted cytotoxics.

Today, the process of small molecule ligand discovery often relies on high-throughput screening procedures with large compound collections (up to 1 million molecules), followed by extensive medicinal chemistry optimization. These activities are not only extremely demanding in terms of time, management, logistics and costs, but often fail to yield hits with sufficient affinity, especially to “difficult” targets (such as those involved in protein-protein interaction).[192, 193]

Alternatively, approaches based on computational design or virtual screening are widely practiced.[194]

In contrast to these technologies, selection methods have been developed (e.g., antibody phage display) which allow the isolation of specific binders (e.g., antibodies) against diverse classes of targets in relatively straightforward in vitro procedures. Essential for this approach is a stable linkage between the potential binding compound (“phenotype”) and the corresponding encoding information (“genotype”). Following incubation of the library with immobilized antigen (“panning”), non-binders are washed off and specific binders recovered by elution. The attached genetic information is amplified and sequenced in order to establish the identity of the binder.[68] The power of such approaches is illustrated by widespread use of phage-display and similar display-approaches.[195, 196]

In the following we will discuss methods based on the principle of genotype-phenotype linkage, which can be used for the identification of low molecular weight binders, more specifically short peptides and non-peptidic small molecules. Since DNA in all of these settings has emerged as the carrier of ligand identity information, these approaches can be summarized as DNA encoded chemical library technologies. We believe that these methods hold great promise for the identification of homing ligands for drug-delivery applications. For one, they do not require the costly infrastructure associated with high-throughput screening thus allowing also academic institutions and smaller companies to work in the field. Secondly, ligands recovered from DNA encoded libraries already have defined conjugation sites, which are required for payload attachment.
Some DNA encoded library technologies have already yielded binders \( M = 500 – 2000 \text{ g mol}^{-1} \) capable of selective tumor accumulation \textit{in vivo}, others are very promising in our view but a definite proof has not been established yet.

4.1. DNA Encoded Libraries of Peptides

Phage-display is widely used for the construction of linear short-peptide libraries. In brief, filamentous phage (\textit{e.g.}, M13) can be engineered to express foreign amino acid sequences fused to certain coat proteins without compromising phage infectivity and propagation ability thus establishing a stable genotype-phenotype linkage.\[^{195, 197}\]

This approach has been used in the identification of linear peptide ligands for various tumor markers and has been covered in recent reviews.\[^{198, 199}\] Although often successful \textit{in vitro}, only few peptides showed adequate targeting performance in tumor models \textit{in vivo}. When bio-distributions of labeled peptides were performed, tumor uptake was often shown to be very low with high accumulation in liver\[^{200}\] or kidneys.\[^{198, 201}\]

Nevertheless, some peptides derived from phage-display have been used for the construction of drug conjugates and tested \textit{in vivo}. A conjugate of the integrin binding peptide RGD-4C with doxorubicin exhibited reduced toxicity and more potent tumor growth retardation than doxorubicin alone in a murine model of cancer.\[^{202}\] A conjugate of an EphA2 binding peptide and paclitaxel connected through an ester linkage has recently been shown to induce a more potent anti-tumor effect than paclitaxel alone in mice.\[^{203}\]

Other peptides have shown some success as imaging agents suggesting potential applications as homing ligands in drug conjugates. The RMS-II peptide, for example, labeled rhabdomyosarcoma lymphatics and blood vessels \textit{in vivo}.\[^{204}\] Similarly, a peptide raised against the prostate cancer marker hepsin\[^{205}\] allowed near infrared fluorescence mediated tomography (FMT) imaging of LNCaP xenografts in mice. The \(^{64}\)Cu-DOTA derivative of AE105 exhibited good (8.1 % ID g\(^{-1}\)) accumulation in U87MG glioblatoma tumors in rodents.\[^{206, 207}\]
Figure III.4: a) Construction of phage libraries of linear or circular peptides in bacteria.\textsuperscript{[195, 197, 208]} (1) Phagemid libraries are assembled by PCR and transfected into \textit{E. Coli}. (2) Superinfection with a helper-phage facilitates expression of phage bound peptides. (3) Cyclization of the phage bound peptides with reactive chemical scaffolds yields the library. b) Synthesis of peptide-libraries containing unnatural amino acids using mRNA-display and \textit{in vitro}-translation.\textsuperscript{[209]} (1) Using whole cell lysate and nonsense-codon suppressing amino acyl-tRNA (aa-tRNA).\textsuperscript{[210, 211]} (2) Using the „\textit{in vitro} translation system from purified components“ (PURE).\textsuperscript{[212, 213]} Unnatural amino acids (green balls) can be incorporated by withdrawing natural amino acids (yellow balls) and adding unnatural ones, which can be charged onto tRNA by natural aa-tRNA synthetases. (3) Using the random non-standard peptide integrated discovery (RAPID) system.\textsuperscript{[214-216]} Selected natural amino acids (yellow balls) and aa-tRNA synthetases (yellow oval shape) are withdrawn from the PURE system and tRNA charged with unnatural amino acids (green balls) is added. The synthesis of unnatural amino acid containing aa-tRNA is conveniently performed using flexizymes (green oval shape) (4) Cyclization of the peptide \textit{e.g.}, through the side chains of unnatural amino acids.

High kidney and liver uptake can compromise the \textit{in vivo} performance of peptide-based delivery approaches. Nonetheless, experience gained from somatostatin analogs and other peptide-based pharmacodelivery strategies suggest that when abundant expression of an accessible target and high binding affinity are given, an efficient and selective tumor uptake is possible.\textsuperscript{[106]} Research efforts are therefore
increasingly focused on conformationally restricted peptides (e.g., cyclic peptides), which pay a lower entropic cost when binding to the cognate target. Phage-display of disulfide-constrained peptides\[^{[219-221]}\], for example, has successfully been used for the identification of cyclic RGD-ligands for different integrins\[^{[222]}\] or a tumor-homing peptide targeting lymphatic vessels.\[^{[223]}\] Sir Gregory Winter and Christian Heinis (Figure III.4a) have developed an approach by which linear peptide precursors containing three Cys residues were cyclized with a trivalent reactive chemical scaffold.\[^{[208]}\] Using this methodology a potent and highly specific inhibitor of human plasma kallikrein \[^{13}\] (Scheme III.5, \(K_i = 1.5 \text{ nM}\))\[^{[208]}\], and an inhibitor for urokinase-type plasminogen activator (uPA), a serine protease that is implicated in tumor growth and invasion (\(K_i = 53 \text{ nM}\)) were identified.\[^{[224]}\] More recently, an improved inhibitor of human plasma kallikrein (\(K_i = 0.3 \text{ nM}\)) was reported.\[^{[225]}\]

Several methods relying on \textit{in vitro} translation systems for the construction of DNA encoded cyclic peptide libraries have also been reported (Figure III.4b). Here, the stable phenotype-genotype linkage is usually obtained through the action of puromycin, which stably links the peptide to its encoding mRNA derived from the DNA library.\[^{[209]}\]

Rabbit reticulocyte lysate has been shown to efficiently translate a library of approximately \(1.2 \times 10^{11}\) peptides linked to their respective encoding mRNAs. Cyclization was achieved through Cys residues encoded in the sequence. Potent \(\alpha\)-thrombin binders with (\(K_D\) values down to 166 nM) could be isolated using this approach.\[^{[210]}\] Using nonsense codon suppression it is further possible to include unnatural amino acids into similar systems.\[^{[211]}\] A very strong cyclic inhibitor of \(G\alpha_{i1}\) (\(K_i = 2.1 \text{ nM}\)) was identified using such an approach. In order to construct libraries capable of simultaneously containing several different unnatural amino acids, a more extensive re-programming of the genetic code has to be contrived. Jack Szostak and co-workers have thus used an \textit{in vitro} translation system reconstituted from purified components (PURE)\[^{[212]}\] in combination with mRNA display to synthesize cyclic peptide libraries containing many different unnatural amino acids. Thrombin binders with \(K_D\) values down to 4.5 nM could be isolated in several rounds of selections.\[^{[213]}\]

The most advanced system for the construction of DNA encoded cyclic peptide libraries containing numerous unnatural amino acids to date is the random non-standard peptide integrated discovery (RAPID) system described by Suga and co-
workers. Essentially relying on the PURE system, the enormous advantage lies in the fact that aa-tRNA synthesis is achieved through a flexible ribozyme (flexizyme)\textsuperscript{[214, 215]} which can charge almost arbitrary amino acids onto tRNAs.\textsuperscript{[216]} Using libraries based on thioether cyclization of the backbone, potent ($IC_{50}$s in the range of 100 nM) and isoform selective inhibitors of Akt2\textsuperscript{[217]} 14 (Scheme III.5) and a potent binder of ubiquitin ligase E6AP ($K_D = 0.60$ nM)\textsuperscript{[218]} could be identified.

\begin{center}
\textbf{Scheme III.5:} Examples of molecules isolated from DNA encoded peptide libraries. Human plasma kallikrein inhibitor 13\textsuperscript{[208]} and Akt2 inhibitor 14.\textsuperscript{[217]} Unnatural structural features are highlighted in blue.
\end{center}

These peptide-based encoded libraries appear to be able to yield binders to a large variety of different protein targets, but their potential for pharmacodelivery applications remains to be fully verified \textit{in vivo}. As these technologies are mainly restricted to the biosynthetic capability of the ribosome, alternative strategies are
needed for the construction of DNA encoded libraries, based on structurally more
diverse small organic compounds.

4.2. DNA Encoded Libraries of Small Organic Molecules

In 1992 Brenner and Lerner proposed in a theoretical paper to encode synthetic
peptides with oligonucleotides (merely acting as amplifiable “bar codes”, rather than
as genes for biosynthesis) on the same solid support.\textsuperscript{[226]} Whilst this article marked
the birth of DNA encoded library synthesis, it was only a decade later that this
concept was implemented into practice in the absence of beads, thus allowing the
construction and screening of libraries of unprecedented size. The stepwise split and
pool synthesis has emerged as a particularly robust strategy for the assembly of DNA
encoded chemical libraries and is currently widely practiced (Figure III.5a).\textsuperscript{[227-232]} In
brief this approach features the following sequential steps:\textsuperscript{[233]} (i) A first set of
chemical compounds is coupled to short DNA fragments coding for the respective
building block. (ii) The products of the first step are pooled and split into separate
reaction vessels. (iii) The second building block is coupled and the reaction encoded
on the DNA tag (e.g., by hybridization of a partially complementary oligonucleotide
followed by Klenow-fragment assisted DNA polymerization). (iv) Pooling of the last
encoding step yields the final DNA encoded chemical library. Steps (ii) and (iii) can in
principle be repeated several times to generate libraries with multiple building blocks
and sizes between $10^5$ and $10^9$ encoded small molecules. In practice, however,
multiple split and pool rounds are unavoidably associated with decreasing library
purities as unwanted side-products accumulate. Also, in spite of the tremendous
progress made in the field of high-throughput DNA sequencing, sampling libraries
containing dozens of millions of compounds remains an expensive task.
Figure III.5: a) DNA encoded library construction by sequential split and pool synthesis using peptide chemistry. (1) Couple building blocks $A_i$ to oligonucleotides coding for $A_i$. Deprotect, purify reactions, pool and split into $m$ aliquots. (2) For each aliquot, react with one building block $B_j$. Precipitate DNA and encode reaction step using Klenow polymerization. Pool all reactions to obtain the final library. b) DNA-templated library synthesis (DTS) as practiced by Liu and coworkers. DNA-bound building blocks (e.g., $C_k$) anneal with complementary sequences on the coding DNA strand. The spatial proximity promotes reaction of $C_k$ with the previous building blocks. Linker cleavage between the building blocks and their coding DNA followed by a workup step finishes the cycle. c) Library construction by DNA-routing. Subsets of the library are sequentially immobilized on beads bearing sequences complementary to coding sequences. Each immobilized sub-library is reacted with the corresponding building block, eluted and mixed with the rest of the library. For this technique, oligonucleotides complementary to all coding sequences of the library need to be available on solid support. d) Library construction by DTS using a "yoctoliter reactor". Here, three-way hairpin junctions promote reaction through spatial proximity. e) Encoded self-assembling chemical (ESAC) libraries. Two sublibraries bearing different building blocks are hybridized through a constant domain allowing the construction of high-quality combinatorial libraries of size $n \times m$ from two sublibraries sized $n$ and $m$. f) Library construction similar to ESAC based on DNA coding strands and complementary PNA bearing the building blocks.

Importantly, DNA encoded chemistry crucially relies on the availability of highly efficient coupling reactions, which are compatible with the structure of DNA and can proceed in the presence of water. Diels-Alder, $\text{Cu}^I$ catalyzed azide-alkyne 1,3-dipolar...
cycloaddition, nucleophilic aromatic substitution, reductive amination and peptide coupling reactions have been used to this end.\footnote{231, 232, 248}

An alternative to manual split and pool assembly of DNA encoded chemical libraries is represented by DNA templated synthesis (DTS). In this strategy, DNA is not only used as a unique identifier for the attached pharmacophore but also to direct the synthetic steps of library assembly. Liu and co-workers reported an approach, which relied on the observation that chemical reactions could be promoted by bringing DNA bound reactants into close proximity through double strand formation (Figure III.5b). Small organic compounds were coupled to biotinylated DNA fragments (“donors”) and were transferred to suitable complementary DNA strands (“acceptors”). The “donor” strand was subsequently cleaved and removed using avidin coated beads.\footnote{234-237}

The group of Harbury described a strategy termed “DNA routing” (Figure III.5c).\footnote{238, 239} DNA strands representing library members are randomly assembled from individual coding segments. Building blocks are then coupled to subsets of the library, which have been selectively immobilized on resins bearing oligonucleotides complementary to the respective coding segments. After washing, DNA elution and pooling the next synthesis step can be performed.

More recently, Hansen and coworkers used three way DNA hairpin junctions to transfer appropriate donor chemical moieties onto a core acceptor site (Figure III.5d).\footnote{240}

The above-mentioned strategies are all characterized by a single molecular entity coupled to a DNA strand and are thus often termed “single pharmacophore libraries”. Alternatively, also library formats presenting two separate molecular entities (“dual pharmacophore libraries”) have been developed. Our group described the construction of encoded self-assembling chemical libraries (ESAC) allowing the combinatorial assembly of encoded sub-libraries through DNA heteroduplex formation (Figure III.5e).\footnote{241-245} To this end, two collections of compounds are attached to the 3’ and 5’ ends of short DNA strands respectively. The DNA tag consists of a constant complementary hybridization domain facilitating duplex formation between the sub-libraries and a variable code representing the individual chemical moieties. Mixing equimolar amounts of every library member, a combinatorial library spontaneously assembles representing every possible combination of building blocks. Since only a single synthetic step is required for
making the sub libraries, very large libraries of high quality can be constructed. Winssinger and co-workers have pursued a conceptually similar approach. Here, molecules conjugated to PNA were allowed to self assemble on a DNA template which could directly be used in PCR amplification after selections (Figure III.5f). In both approaches, the flexibility of the linkers between DNA and the displayed molecules allows the simultaneous exploration of two different binding pockets on the same target protein yielding a significantly increased binding affinity due to the chelate effect. After identification of potential binders, the two binding compounds have to be arranged into one molecule similar to fragment based drug discovery approaches. It is worth mentioning that DNA encoded chemical libraries can not only be used for the de-novo discovery of small molecule binders of target proteins but also as a tool for the systematic exploration of binding space around a known lead compound (“affinity maturation” or “lead expansion”).

DNA encoded chemical libraries are now being actively used in hit and lead discovery programs in both academic and industrial settings. Indeed, binders and inhibitors of many targets of pharmaceutical interest have been identified using this approach. Our group has recently demonstrated that DNA encoded chemical libraries can be used for the efficient discovery of ligands which selectively target neoplastic tissue. Carbonic anhydrase IX (CAIX) is a membrane bound enzyme, which is overexpressed in many forms of solid tumors either in response to hypoxia or when the van Hippel Lindau tumor suppressor protein (pVHL) is inactivated by mutation. A ligand of CAIX isolated from a 1-million member DNA encoded chemical library 15 (Figure III.6a, inhibits p-nitrophenyl acetate hydrolysis by CAIX with $IC_{50} = 260$ nM) selectively accumulated in the tumor, upon injection in a mouse model of human cancer (Figure III.6b). We and others are currently working on improved CAIX targeting agents, for the delivery of potent cytotoxic molecules to renal cell carcinoma.
Figure III.6: a) Fluorescein-conjugate of a CAIX ligand 15, which was isolated from a 1-million member DNA encoded chemical library, accumulates in solid tumors in mice after intravenous injection. b) Photographs and fluorescence pictures of extracted organs. (1) tumor, (2) liver, (3) lung, (4) spleen, (5) heart, (6) kidney.

5. Cytotoxic Payloads and Linker Technologies
Cytotoxic payloads for targeted delivery applications (Table III.1) should ideally exhibit a very high activity (the number of ligand-drug conjugates which can be delivered to a cell is inherently limited by the number of antigens expressed on its surface) and must have a site amenable to ligand attachment. Most classes of cytotoxic drugs currently used in the clinic have been tested as conjugates including methotrexate, vinca-alkaloids, taxoids and anthracyclines. In spite of their relatively low toxicity, early attempts often relied on doxorubicin and its derivatives and methotrexate as their chemical structures were permitting facile modification and the drugs were available in large quantities. Nowadays much more potent payloads are preferred. Importantly, the prodrug nature of targeted drug conjugates allows the use of cytotoxic agents, which are otherwise too potent to be given for therapy. The most widely used payloads currently are dolastatin analogs such as the auristatins and cemadotin, maytansinoids,
calicheamicins\textsuperscript{[252, 263]}, pyrrolobenzodiazepines\textsuperscript{[253, 264]}, duocarmycins\textsuperscript{[254, 265]} and tubulysins.\textsuperscript{[102, 142, 143, 255, 266]}

<table>
<thead>
<tr>
<th>Compound</th>
<th>Family</th>
<th>Min</th>
<th>Max</th>
<th>Median</th>
<th>Cell Lines Tested</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>methotrexate\textsuperscript{[a]}</td>
<td>antifolates</td>
<td>$2.4 \times 10^{-8}$</td>
<td>$2.5 \times 10^{-8}$</td>
<td>$7.3 \times 10^{-8}$</td>
<td>97</td>
<td>[251]</td>
</tr>
<tr>
<td>doxorubicin\textsuperscript{[a]}</td>
<td>anthracyclines</td>
<td>$2.5 \times 10^{-8}$</td>
<td>$1.3 \times 10^{-8}$</td>
<td>$8.4 \times 10^{-8}$</td>
<td>85</td>
<td>[251]</td>
</tr>
<tr>
<td>paclitaxel\textsuperscript{[a]}</td>
<td>taxoids</td>
<td>$2.8 \times 10^{-9}$</td>
<td>$1.6 \times 10^{-9}$</td>
<td>$9.6 \times 10^{-9}$</td>
<td>94</td>
<td>[251]</td>
</tr>
<tr>
<td>vinblastine\textsuperscript{[b]}</td>
<td>vinca alkaloids</td>
<td>$2.5 \times 10^{-10}$</td>
<td>$2.7 \times 10^{-10}$</td>
<td>$6.4 \times 10^{-10}$</td>
<td>99</td>
<td>[251]</td>
</tr>
<tr>
<td>maytansine\textsuperscript{[b]}</td>
<td>maytansinoids</td>
<td>$1.2 \times 10^{-9}$</td>
<td>$2.0 \times 10^{-9}$</td>
<td>$3.3 \times 10^{-9}$</td>
<td>74</td>
<td>[251]</td>
</tr>
<tr>
<td>duocarmycin SA\textsuperscript{[b]}</td>
<td>duocarmycins</td>
<td>$3.2 \times 10^{-11}$</td>
<td>$6.8 \times 10^{-11}$</td>
<td>$7.1 \times 10^{-11}$</td>
<td>76</td>
<td>[251]</td>
</tr>
<tr>
<td>auristatin PE\textsuperscript{[b]}</td>
<td>dolastatins</td>
<td>$8.7 \times 10^{-11}$</td>
<td>$1.0 \times 10^{-11}$</td>
<td>$4.1 \times 10^{-11}$</td>
<td>60</td>
<td>[251]</td>
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<tr>
<td>SJG-136</td>
<td>calicheamicins</td>
<td>&lt;5.0 x 10^{-11} in HeLa cells</td>
<td></td>
<td></td>
<td>1</td>
<td>[252]</td>
</tr>
<tr>
<td>methotrexate\textsuperscript{[a]}</td>
<td>antifolates</td>
<td>$2.3 \times 10^{-12}$</td>
<td>$5.0 \times 10^{-12}$</td>
<td>$4.7 \times 10^{-12}$</td>
<td>8</td>
<td>[253]</td>
</tr>
<tr>
<td>duocarmycin SA\textsuperscript{[b]}</td>
<td>duocarmycins</td>
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<td>$5.7 \times 10^{-13}$</td>
<td>$6.2 \times 10^{-13}$</td>
<td>15</td>
<td>[254]</td>
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<tr>
<td>tubulysin D</td>
<td>tubulysins</td>
<td>$3.1 \times 10^{-12}$</td>
<td>$6.7 \times 10^{-12}$</td>
<td>$8.9 \times 10^{-12}$</td>
<td>4</td>
<td>[255]</td>
</tr>
</tbody>
</table>

\textsuperscript{[a]} The current dataset (03/2012) of the National Cancer Institute’s (NCI) drug screening database was used. For each cell line averages were calculated from all test results available for the indicated compound.

\textsuperscript{[b]} Absolute IC\textsubscript{50} values were estimated from Figure 1 of the corresponding reference. Only human cancer cell lines were included in the analysis.

**Table III.1:** Growth inhibition data of cytotoxic agents representing the most common payload families. The IC\textsubscript{50} values for the most sensitive cell lines (Min), least sensitive cell lines (Max) and the median IC\textsubscript{50} values are given.

The cytotoxic drug should remain stably attached to the targeting ligand whilst the conjugate is in circulation but be released efficiently when the construct reaches the intended site of action. Linkages undergoing pH-dependent\textsuperscript{[139, 260, 263]}, reductive\textsuperscript{[57, 127, 267]} and enzyme catalysed cleavage\textsuperscript{[268, 269]} have been developed towards this aim (Table III.2). When suitable functional groups are present directly on the ligand and payload (e.g., thiols) a “linkerless” connection can be established facilitating “traceless” release of the drug (Scheme III.6a).\textsuperscript{[57, 260]} When this is not possible cleavable linkers are required as adaptors to connect the ligand to the payload (Scheme III.6b). In this case, ligands or drugs may be released with covalent modifications\textsuperscript{[270]}, which can potentially complicate pharmacokinetics or give rise to immunogenicity. Most linkage chemistries discussed here were originally developed for the connection of antibodies to cytotoxic payloads but can in principle be used without modification with other targeting vehicles.
Table III.2: Commonly used cleavable linkages for the construction of ligand-targeted cytotoxics

<table>
<thead>
<tr>
<th>Linkage</th>
<th>Structure</th>
<th>Release Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>amide</td>
<td><img src="image" alt="amide" /></td>
<td>hydrolysis</td>
</tr>
<tr>
<td>ester</td>
<td><img src="image" alt="ester" /></td>
<td>hydrolysis</td>
</tr>
<tr>
<td>carbamate</td>
<td><img src="image" alt="carbamate" /></td>
<td>hydrolysis</td>
</tr>
<tr>
<td>hydrazone</td>
<td><img src="image" alt="hydrazone" /></td>
<td>hydrolysis</td>
</tr>
<tr>
<td>thiolidzone</td>
<td><img src="image" alt="thiolidzone" /></td>
<td>hydrolysis</td>
</tr>
<tr>
<td>disulfide</td>
<td><img src="image" alt="disulfide" /></td>
<td>reduction</td>
</tr>
</tbody>
</table>

Initial attempts of producing targeted drug conjugates often relied on dicarboxylic acid spacers coupled to the drug and the targeting vehicle as esters, amides or carbamates.\[111-113, 123\] If such constructs reach the acidic environment of the lysosome, drug release by spontaneous spacer hydrolysis may occur. Alternatively, cleavage of the amide or ester bond by lysosomal proteases or esterases can be envisioned. Experimental evidence for the exact mechanism of drug-release and its efficiency, however, is often lacking. It should nevertheless be noted that peptide-drug conjugates in which amide bonds or carbamates connect the targeting vehicle to the payload often exhibit lower potency than the free drug in vitro\[103, 123, 124, 271\] suggesting inefficient cleavage. Interestingly, antibody drug conjugates (ADCs) based on very stable linkages (e.g., in the form of spacers attached to the antibody through a maleimide and to the drug as an amide) have shown remarkable potency and have been progressed through clinical trials.\[267\] It is hypothesized that the entire antibody is proteolytically degraded once inside the lysosome thus releasing the payload. More recently, peptide spacers such as the valine-citrulline linker have been designed with specific cleavage sites for lysosomal proteolytic enzymes and are now widely being employed.\[268, 269\]

Hydrazones are acid labile and hydrolysed in the lysosome and certain endosomes.\[139, 263\] Our group has recently proposed the use of thiazolidines as an acid labile traceless linkage. In this case, an aldehyde-containing drug is reversibly
conjugated to a 1-thio-2-aminoethyl motif on the targeting vehicle such as an N-terminal Cys residue on certain antibody formats.\cite{260}

Disulfide linkages are commonly thought to undergo reductive cleavage in the endosome upon internalization and indeed some direct evidence for this process has been provided.\cite{127} In their purest form, they can be established between thiols on the drug (e.g., cemadotin-SH) and the targeting vehicle\cite{57} facilitating a traceless separation between carrier and payload. Until now, however, alkyl spacers with disulfide groups are often used.\cite{66, 67, 260} Their release kinetics can be modulated by the steric bulk around the disulfide bond.\cite{267} When the cytotoxic payload does not tolerate attachment of an adaptor without losing activity, self-immolative linker systems based on 2-mercaptoethyl carbonates and carbamates\cite{177} or 2-mercaptophenylacetate\cite{272} can be used (Scheme 6c). Traceless drug linkage technologies may be preferable, though, as the elimination of self-immolative linkers is not an instantaneous process.

\[ \text{ligand} \text{S} \text{S} \text{O} \text{O} \text{R} \text{S} \text{S} \text{O} \text{O} \text{H} \text{N} \text{N} \text{H} \]

\[ \text{ligand} \text{N} \text{H} \text{O} \text{S} \text{S} \text{O} \text{O} \text{n} \text{m} \text{O} \text{H} \text{N} \text{O} \]

\[ \text{ligand} \text{R} \text{S} \text{S} \text{O} \text{O} \text{H} \text{N} \text{O} \text{RS} \text{S} \text{O} \text{O} \]

\[ \text{ligand} \text{O} \text{RS} \text{S} \text{O} \text{O} \text{H} \text{N} \text{O} \text{RS} \text{S} \text{O} \text{O} \]

\textbf{Scheme III.6:} a) Traceless disulfide linkage.\cite{57} b) Disulfide linker connecting ligand and payload with free amines.\cite{270} c) Self-immolative linkers based on disulfides as the cleavable linkage.\cite{177, 272}

The exact conditions encountered by a ligand-drug conjugate seem to be dependent on the tumor antigen targeted by the delivery vehicle. Folate receptors for example do not seem to internalize into acidic endosomes or the lysosome.\cite{127, 163} Similarly, HER2 internalization does not efficiently facilitate cleavage of disulfide linkers at least in certain cell lines.\cite{273} A good understanding of the endocytotic pathway of the antigen under investigation thus appears to be critical for the correct choice of linkage technology.
6. Remaining Challenges and Outlook

Ligand-based delivery of cytotoxic drugs shows great potential in the pharmacotherapy of cancer and other serious conditions. Small molecule and peptide homing vehicles are already available for a number of attractive biomarkers (e.g., tumor-associated receptors, proteases, phosphatases and carbonic anhydrases) and their conjugates have shown selective accumulation and therapeutic benefit in in vivo experiments.

Nevertheless, many challenges still remain to be solved. Using current technologies, it is still difficult to isolate specific ligands to some of the most attractive accessible markers of pathology (e.g., components of the modified extracellular matrix). In the case of antibody phage-display, it has been shown that the use of library subsets with only millions of antibodies does not typically yield high affinity binders (or yield binding specificities at all). Libraries of billions of antibodies on the other hands yielded binders against a large variety of targets. The construction and use of larger and better DNA encoded chemical libraries will enhance the performance of this technology for ligand-discovery and pharmacodelivery applications.

The side-by-side comparison of antibody-based targeting of disease and of the corresponding pre-targeting strategies reveal a striking benefit associated with small organic ligands, in terms of rapid tissue distribution, efficient uptake at site of disease and rapid clearance. While important for mechanistic studies, pre-targeting technologies are difficult to implement in the industrial and clinical setting, due to their multi-component nature and to the requirement of precise timing between successive injections. Small targeted cytotoxics, consisting of potent drugs directly coupled to good-quality ligands, promise to be more efficient and easier to develop.

All molecules injected into patients, at some stage, need to be removed from circulation. Indeed, in the absence of metabolic degradation, virtually 100% of injected drugs end up, at some time point, in clearance organs (most importantly, liver or kidney). The differential damage inferred to the diseased cells (e.g., to solid tumors) and to normal cells depends on many parameters, including targeting efficiency, drug release kinetics and mechanisms, as well as the inherent sensitivity of different organs to the action of cytotoxic drugs. In the future, it would be desirable to design targeted cytotoxic drugs, which are inactivated during the clearance process (e.g., ester cleavage by liver esterases) and may thus help spare normal organs.
Intense research activities are exploring various classes of cytotoxic drugs and linker strategies. Many potent drugs target tubulin (thus impacting on cell division)[275], but these agents may be less effective against dormant tumor cells. For this reason, DNA-alkylators and other classes of cytotoxic drugs are increasingly being considered for pharmaceutical applications, primarily in the oncology area. In some cases, the biochemical drug target remains to be elucidated in detail. For example, duocarmycins, which were thought to mainly act as minor groove intercalators and as DNA-alkylating agents, have recently been shown to bind to aldehyde dehydrogenases, which represent some of the best studied examples of cancer stem cell markers.[276-278]

In cancer chemotherapy, cytotoxic drugs are commonly believed to mainly impact on tumor cells. However, in principle, profound anti-cancer effects could also be achieved by the selective destruction of a subset of target cells. In the future, it will be important to learn how the modulation of vascular properties, the action of stem cells and the regulation of immune response may contribute to the success or failure of a pharmacological intervention. Indeed, the mechanism of action of targeted drugs may be more complex than the simple killing of diseased cells, which bear the cognate antigen on their surface. In addition to a documented by-stander effect on neighboring tumor cells[279], the diffusion of potent cytotoxic drugs may have an impact on tumor endothelial cells, potentially triggering blood vessel thrombosis and an avalanche of tumor cell deaths[280] or on leukocytes (potentially affecting the action of subsets of immune cells, such as regulatory T-cells and cytotoxic T-lymphocytes).

Finally, we anticipate that the use of non-invasive sensitive imaging methodologies (e.g., positron emission tomography) will be increasingly important to evaluate the targeting performance, facilitating patient selection and the optimization of cytotoxic drugs. We believe that advances in ligand identification from large combinatorial libraries will have a rapid impact on anti-cancer research, as many of the targets, linkers and drugs which are currently used with antibody vehicles should be readily adapted to work in the context of fully synthetic, next-generation targeted cytotoxics.
IV. CARBONIC ANHYDRAZE IX AS A TARGET FOR CANCER THERAPY

1. Carbonic Anhydrase IX Expression in Solid Tumors

Carbonic anhydrase IX (CAIX) is a tumor cell surface antigen, which is constitutively expressed in >80% of renal cell carcinoma.\(^{[281]}\) It is among the best-characterized cell-surface markers of this disease.\(^{[282]}\) In many other solid tumors including glioblastoma\(^{[283]}\), colorectal\(^{[284]}\) and breast cancer\(^{[285]}\), CAIX is expressed in response to hypoxia and plays an important role in tumor acid-base homeostasis by catalyzing the equilibrium between \(CO_2\) and carbonate.\(^{[184, 286]}\) While in hypoxic tumors it is often considered as a marker of poor prognosis\(^{[287]}\), CAIX-expression has been suggested to be positively correlated with survival in renal cell carcinoma patients receiving interleukin-2 (IL-2) treatment.\(^{[288]}\)

![Figure IV.1: Regulation of the expression of CAIX: Under normoxic conditions, hypoxia inducible factor (HIF) 1α (grey) is hydroxylated with molecular \(O_2\) on several proline residues, recognized by the von the von Hippel-Lindau tumor suppressor protein (VHL, blue) and marked for ubiquitin dependent proteasomal degradation. Under hypoxic conditions or when VHL is deactivated by mutation, HIF-1α translocates to the nucleus and pairs up with HIF-1β (green) to drive expression hypoxia response element (HRE, blue box) regulated genes including CAIX.](image)

Expression of CAIX in renal cell carcinoma\(^{[289]}\) and hypoxic tumors\(^{[58]}\) is believed to be regulated by hypoxia inducible factor 1 (HIF-1, Figure IV.1). Under normoxic conditions, HIF-1α is hydroxylated on proline residues 564 and 402 in a process requiring molecular oxygen and primed for ubiquitin-mediated degradation by the von Hippel-Lindau tumor suppressor (VHL).\(^{[58, 290]}\) Also hydroxylation on proline 567 has been proposed.\(^{[290]}\) Under hypoxia (i.e., when oxygen is lacking) hydroxylation does not take place and HIF-1α can pair up with HIF-1β to activate the transcription of diverse downstream genes such as CAIX.\(^{[58]}\) Alternatively, HIF-1α degradation is also blocked when the VHL protein is disabled by mutation. VHL mutations are common
in renal cell carcinoma\cite{291,292} and are widely believed to be responsible for the high proportion of CAIX-expression in this type of tumor.\cite{293} Other studies have suggested that VHL mutations alone cannot explain this expression pattern.\cite{294}

Other carbonic anhydrase isoforms fulfill diverse physiological roles in the body such as bicarbonate transport in the kidneys\cite{295}, or transport of CO$_2$ in blood.\cite{296} While many are intracellular (e.g., the abundant CAII isoform), some membrane-bound carbonic anhydrases are also accessible from the extracellular environment (e.g., CAXII).\cite{297,298}

### 2. Inhibition of Carbonic Anhydrase IX as an Anticancer Strategy

As already mentioned above, CAIX can be detected in various types of solid tumors in response to hypoxia and is believed to play an important role in intratumoral acid-base homeostasis.\cite{165} It has been reported that inhibition of CAIX activity with small organic molecules can retard tumor growth\cite{299}, inhibit metastasis\cite{300} and cancer stem cell expansion in breast cancer in mice.\cite{287} Our group has previously demonstrated a modest growth retardation effect in the SKRC52 xenograft model of renal cell carcinoma in mice using a derivative of the CAIX-inhibiting drug acetazolamide.\cite{301} It remains to be seen in how far these results can be translated into the clinic.

A large number of carbonic anhydrase ligands has been developed, in particular thanks to the efforts of Claudiu Supuran and his group.\cite{302} Typically, inhibitors consist of an aromatic sulfonamide for example of the form 1-4, which directly binds to the conserved Zn$^{2+}$ ion in the active site of the carbonic anhydrase enzyme. Substituents on the aromatic ring can further improve binding and impart at least some isoform selectivity. A selection of commonly used aromatic sulfonamide scaffolds are shown in Scheme IV.1. Binding affinities are typically very high ($K_D < 100$ nM for many ligands and carbonic anhydrases) but good isoform selectivity is hard to obtain due to the large sequence homology within the carbonic anhydrase family.\cite{303} Also sulfamates of the form 5 and 6 (X = O), and sulfamides of the form 6 (X = NH)\cite{304,305} and coumarins (7)\cite{306} have been investigated as ligands.
Scheme IV.1: Different carbonic anhydrase inhibitor chemotypes: aromatic sulfonamides 1-4, cyclic sulfamate 5, sulfamate (X = O) and sulfamide (X = NH) 6 and coumarins (7)

3. Carbonic Anhydrase IX as a Target for Drug Delivery

Several groups have tested the capacity of monoclonal antibodies against CAIX to accumulate in antigen-positive tumors. The group of Otto Boerman has reported spectacular results with its G250 antibody in different models of renal cell carcinoma achieving tumor accumulations of up to 250 % ID g⁻¹ with the antibody alone and over 300 % ID g⁻¹ in a pretargeting approach. Pastorekova and co-workers have shown that the M75 anti-CAIX antibody could reach HT29 colorectal cancer xenografts with an accumulation of up to 15 % ID g⁻¹. Interestingly, our own group could not see strong accumulation of high-affinity (K_D = 2.4 nM for the scFv) anti-CAIX monoclonal antibodies in small immunoprotein (SIP) format derived from phage-display in SL147T colorectal cancer xenografts. Reformatting to IgG did not change the maximum achieved uptake (unpublished results). While it is not straightforward to reconcile these contrasting findings, these results suggest that it should be possible to target CAIX-expressing tumors with monoclonal antibodies. However, the efficiency of the process may strongly depend on the nature of the antibody and the cognate epitope.

Bayer has been working on an antibody drug conjugate (ADC) consisting of an anti-CAIX monoclonal antibody and the auristatin MMAE as the payload. The ADC exhibited a potent antitumor effect in strongly CAIX-expressing tumors. The development status of the product is currently unknown.

4. Unmet Medical Need and Potential in Renal Cell Carcinoma

Kidney cancer kills more than 140,000 people each year worldwide. More than 90% of cases are cancers originating from the kidney itself rather than surrounding tissue and are termed renal cell carcinoma.
Currently available therapies have limited efficacy in particular in a metastatic setting. The tyrosine kinase inhibitors sorafenib (8)\textsuperscript{[310]}, sunitinib (9)\textsuperscript{[311]}, pazopanib (10)\textsuperscript{[311]} and axitinib (11)\textsuperscript{[312]}, which block vascular endothelial growth factor receptor (VEGFR) kinase and platelet-derived growth factor receptor (PDGFR) kinase amongst other targets, and the mammalian target of rapamycin (mTOR) inhibitors temsirolimus (12)\textsuperscript{[313]} and everolimus (13)\textsuperscript{[314]} have been approved. Furthermore IL-2\textsuperscript{[315]}, interferon-\(\alpha\)\textsuperscript{[316]} and the antiangiogenic vascular endothelial growth factor (VEGF)-binding antibody bevacizumab are being used.\textsuperscript{[317]}

Scheme IV.2: Clinically approved agents for the treatment of metastatic renal cell carcinoma; structures were obtained from PubChem and ChEMBL databases.

Only IL-2 has been shown to lead to long-term survival in 6-10% of patients.\textsuperscript{[315, 316]} The remaining agents typically halt disease progression for several months (e.g., 5 months progression-free survival for interferon-\(\alpha\), 11 months for sunitinib in metastatic clear cell renal cell carcinoma).\textsuperscript{[36]} When the patient relapses, switching therapy may give an additional benefit (e.g., 16.6 months overall survival with sorafenib in patients who had previously relapsed on sunitinib).\textsuperscript{[313]} Although termed “targeted therapies”, these treatments are commonly associated with severe side effects.\textsuperscript{[36, 313]} There consequently exists a pressing need for the development of more tolerable and more efficacious drugs for the management of renal cell carcinoma. As a disease that is frequently associated with CAIX expression\textsuperscript{[281, 282]} it is a particularly interesting target indication to be explored in conjunction with therapies directed
against CAIX. Indeed, different laboratories including our own are pursuing this avenue using naked antibody-based\cite{318}, targeted delivery\cite{319,320} and direct enzyme inhibition approaches.\cite{301} So far, none of these molecules has made it to the market suggesting that further work is required to understand how to best exploit CAIX as a therapeutic target in the context of renal cell carcinoma.
V. MONOVALENT SMALL MOLECULE-DRUG CONJUGATES DIRECTED AGAINST CARBONIC ANHYDRASE IX


1. Introduction

The targeted delivery of highly potent cytotoxic agents into diseased tissues has emerged as a promising strategy for the treatment of cancer and other serious conditions. By attaching a therapeutic effector through a cleavable linker to a ligand specific to a marker of disease, the effector preferentially accumulates and acts at the intended site of action, thus increasing the effectively applied dose while reducing side effects. To date, monoclonal antibodies have been considered as the ligands of choice\[67, 79\] and, indeed, research in the field of antibody-drug conjugates (ADCs) has led to the recent approval of two ADCs for applications in oncology: brentuximab vedotin and trastuzumab emtansine.[321]

In spite of these very encouraging developments, the limitations of using antibodies for drug delivery applications are becoming increasingly clear.[38] Antibodies are large macromolecules and thus often have difficulties penetrating deeply into solid tumors.[65] In addition, they can be immunogenic[322] and typically long circulation times[89] can lead to premature drug release and undesired side effects. Moreover, the production of ADCs is expensive, reflecting the need for clinical-grade manufacturing of antibodies, drugs and the resulting conjugates.[38]

The use of smaller ligands as delivery vehicles such as peptides or small drug-like molecules could potentially overcome some of the abovementioned problems. Their reduced size should aid tissue penetration, they should be non-immunogenic and amenable to classic organic synthesis thus reducing manufacturing costs.[38, 323]

Indeed, the favorable properties of drug conjugates using folic acid[141] or ligands against prostate-specific membrane antigen (PSMA)[324] as delivery vehicles have
been demonstrated and a folate conjugate has recently entered Phase III clinical studies. In spite of rising interest from both academia and industry, the field is still in its infancy as few ligands have been studied systematically and only a small number of targets have been drugged successfully.

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

**Figure V.1:** a) Chemical structures of ligand-linker-dye conjugates synthesized for in vitro binding and in vivo targeting studies b) Determination of $K_D$ of 1a-3a, 5a and 6a towards recombinant CAIX extracellular domain in vitro by fluorescence polarization. The $K_D$ of the diazo-ligand 4a could not be determined due to its dark quenching properties. Data points are given as averages of three experiments. Error bars indicate standard deviations. $K_D$ values are given in brackets in nm ± standard errors of fit.

### 2. Results

Based on known structure activity relationship data, a series of high-affinity CAIX ligand-linker-dye conjugates 1a-5c (Figure V.1a) was prepared. Dissociation constants of 1a-3a and 5a towards recombinant CAIX were determined by
fluorescence polarization (FP, Figure V.1b). Flow cytometry experiments indicated ligand-dependent binding of Alexa546 and IRDye750 conjugates 1b and 1c-5c to CAIX-positive cells, but not to control cell lines lacking CAIX (Figures V.2-5).

**Figure V.2:** Cell surface expression of CAIX on different cell lines used in this study analyzed by flow cytometry. aCAIX refers to the Santa Cruz biotechnology polyclonal rabbit anti human CAIX antibody. 2° antibody refers to a suitably labeled anti rabbit IgG antibody for detection. a) SKRC52 cells constitutively express high levels of CAIX. b) A549 cells express only very low levels of CAIX under normoxic conditions. Since they maintain strong attachments to culture plates, these cells are suitable as negative controls in experiments requiring multiple washing steps of attached cells. c) HEK cells do not express detectable levels of CAIX under normoxic culture conditions. They can easily be detached from culture plates with EDTA and were thus used as negative controls in most flow cytometry experiments.

**Figure V.3:** Flow cytometry analysis of binding of ligand-Alexa546 conjugates 1b and 6b to CAIX-expressing SKRC52 and HEK cells lacking CAIX on their cell surface. Cells were detached with EDTA, treated with 30 nM dye conjugate for 1 h at 0 °C, washed and analyzed. a) Only the conjugate bearing a ligand for CAIX can bring about a shift in fluorescence intensity to the right. The conjugate lacking the ligand does not give rise to a shift relative to untreated cells. b) In the absence of a cell surface receptor none of the conjugates can bring about a shift in fluorescence intensity to the right.
Figure V.4: Flow cytometry analysis of binding of ligand-IRDye750 conjugates 1c-6c (Figure V.1a) to CAIX-expressing SKRC52 cells. Cells were detached with EDTA, treated with 30 nM dye conjugate for 1 h at 0 °C, washed and analyzed. Only 1c binds strongly enough to result in a strong fluorescence shift after washing of cells. Given their higher $K_D$, conjugates 2c-5c may dissociate too quickly to be detected. Conjugate 6c, which lacks a ligand for CAIX, only shows little residual binding.

In contrast to previous reports that suggested receptor-based internalization of CAIX-specific ligands\textsuperscript{[328-331]}, acetazolamide-based fluorophore conjugates were found to preferentially bind to the cell membrane of kidney cancer cells without efficient internalization (Figure V.6a), while the same cells were not stained by fluorophores lacking the tumor-homing moiety (Figure V.6b). These results collectively suggest that fluorescent probes 1a-c derived from the approved anti-glaucoma drug acetazolamide (AAZ) were high-affinity CAIX binders ($K_D = 12.6$ nm for 1a) and, thus, potentially suitable for pharmacodelivery applications.
Figure V.5: Flow cytometry analysis of binding of ligand-IRDye750 conjugates 1c-6c (Figure V.1a) to CAIX-negative HEK cells. Cells were detached with EDTA, treated with 30 nM dye conjugate for 1 h at 0 °C, washed and analyzed. In the absence of a specific binding interaction, there is little difference between cells treated with CAIX ligand-dye conjugates and untreated cells.

Figure V.6: a) Confocal microscopy image of CAIX-expressing SKRC52 cells after exposure to targeted dye conjugate 1b (30 nM) for 1h. The conjugate is mainly bound to the cell surface. b) Confocal microscopy image of CAIX-expressing SKRC52 cells exposed to untargeted dye conjugate 6b (30 nM) for 1h. No cell surface binding can be detected. Scale bar indicates 35 µm.
**Figure V.7:** Investigation of internalization of targeted dye conjugate 1c by flow cytometry a) CAIX-positive SKRC52 cells attached to culture plates were incubated with medium containing 30 nM 1c for 1 h at 37 °C. Detachment with trypsin resulted in cells with higher fluorescence intensity than cells treated with non-binding conjugate 6c or untreated cells. b) Aliquots of the same cells which had been treated with 1c and detached with trypsin were stained with an anti CAIX antibody (aCAIX AB) followed by an Alexa488 labeled secondary antibody (2° AB) at 0 °C which upon flow cytometry analysis gave a superimposable histogram to cells treated with secondary antibody only. We concluded that trypsin treatment had removed all surface bound CAIX and the fluorescence shift of 1c labeled cells in a) must come from internalized conjugate. Cells detached from solid support using EDTA and stained as before gave rise to a 10× shift in fluorescence intensity to the right compared to baseline giving us confidence that CAIX detection by flow cytometry did indeed work. c) To further support the claim that we were observing active uptake processes, we decided to repeat the experiment under conditions inhibiting uptake. SKRC52 cells were pre-treated with medium containing 0.2% w/v NaN₃ for 1 h before incubation with 30 nM 1c and 0.2% w/v NaN₃ at 37 °C for 1 h. NaN₃ is known to be an inhibitor of active uptake processes[332] and indeed fluorescent signal was shifted to baseline. The same effect was achieved when incubating with 1c in the presence of excess AAZ as a competitive ligand or when incubating at 0 °C, which also inhibits active uptake processes.
Figure V.7 continued: d) Extending the incubation time with 30 nM 1c at 37 °C from 1 h to 2 h and 4 h we did not see a markedly increased signal. We thus concluded that although some internalization takes place, it is inefficient over time. e) Active uptake of CAIX binding conjugate 1c into CAIX negative A549 cells was tested as described in Figure V.7. Since no shift in fluorescence intensity over baseline was observed, it was concluded that 1c was not taken up into A549 cells. This is expected in the absence of a cell surface receptor for 1c.

We thus investigated the potential of 1b and 1c to to reach tumors in vivo (Figure V.8-10). An intravenous dose of 3 nmol AAZ-based conjugate 1c preferentially accumulated in subcutaneous CAIX-expressing SKRC52 tumors\(^{[94]}\) in nude mice (Figure V.8). Intravenous administration of 3 nmol of 2c resulted in moderate visible tumor accumulation after 1 h, but the tumor to background ratio was only poor (Figure V.9). By contrast, conjugates 3c-5c and untargeted dye conjugate 6c did not exhibit a preferential tumor homing (Figure V.9, Figure V.10). Since 1a has the lowest \(K_D\) value followed by 2a, these results suggest that binding affinity contributes to efficient tumor targeting. As the best cancer localization results were obtained with 1c, we decided to quantitatively evaluate the biodistribution of this compound, comparing it to the tissue distribution properties of the untargeted dye 6c.
**Figure V.8:** Representative near infrared images of nude mice bearing subcutaneous SKRC52 xenograft tumors injected with different doses of targeted IRDye750 conjugate 1c. Already 1 h after the intravenous injection of 1 nmol 1c the tumor can clearly be seen against background. The injection of 3 nmol gives a stronger and longer lasting signal with good tumor to background contrast at early time points and was thus used for further imaging studies. A dose of 10 nmol saturates the fluorescence detector with the parameters used at early time points but leads to an even longer lasting signal.

**Figure V.9:** Representative near infrared images of SKRC52 xenograft bearing mice 1-12 h after intravenous injection of 3 nmol ligand-IRDye750 conjugates 1c-5c and untargeted conjugate 6c as negative control (see Scheme V.1 for structures). Images of whole animals after 24 h are not shown; for extracted organs after 24 h see Figure V.10. Only the AAZ conjugate 1c gave good tumor to background contrast and was thus selected as a basis for further development of a targeted conjugate. After administration of 2c the tumor is barely visible; all other conjugates do not reach the tumor in levels above background fluorescence. Untargeted conjugate 6c also does not reach the tumor and is also cleared faster from the animal than ligand-IRDye750 conjugates.
Figure V.10: Near infrared images of organs extracted 24 h after injection of dye conjugates 1c-6c from mice imaged in Figure V.9 using a 25× higher sensitivity. Tmr = tumor, liv = liver, kid = kidneys, int = intestine, lng = lung, hrt = heart, spl = spleen, mus = muscle, bld = 100 µL of blood in a clear Eppendorff tube. Qualitatively, a decrease in targeting performance from 1c to 5c can be observed and very little tumor or organ accumulation from untargeted conjugate 6c. 1c exhibits the best cell surface binding profile by flow cytometry (Figure V.4), the corresponding FITC conjugate 1a exhibits the best in vitro $K_D$ as measured by fluorescence polarization. 2a ranks second best, 3c third etc. This suggests that binding affinity of the targeting ligand for CAIX is an important determinant for accumulation inside the tumor and in vitro profiling of dye conjugates by FP and flow cytometry has predictive value for in vivo targeting performance.

Figure V.11: Evaluation of tumor-homing performance of targeted conjugates 1c compared to untargeted controls 6c a) Whole-animal near-IR fluorescence images of a representative mouse 1, 2 and 4 h after intravenous administration of 3 nmol targeted ligand-IRDye750 conjugate 1c or 6c. A preferential accumulation of construct 1c in the tumor can be observed. Without the targeting ligand the dye conjugate 6c can only be seen in the kidneys as a major route of excretion. b) Quantification of targeted conjugate 1c or 6c in homogenized samples of tumors extracted 1, 2 or 4 h after intravenous administration of 3 nmol dye conjugate. Error bars indicate standard deviations. Averages of three experiments are shown.

Accumulation of 1c in the tumor was rapid and efficient with $13.4 \pm 3.0 \%$ of injected dose per gram of tissue (% ID g$^{-1}$) after only 1 h (Figures V.11-13). This result
compares favorably with previous work on antibody-based targeting of CAIX expressing tumors, where only markedly lower tumor uptake values (a maximum of \(2.4 \pm 0.2 \% \text{ID g}^{-1}\)) could be detected\(^{[308]}\). In our case, the dye conjugate 1c, however, progressively dissociated from the tumor (residence \(t_{1/2} \approx 1\) h, Figure V.11b), suggesting that an improvement of CAIX binding affinity may further contribute to efficient tumor targeting performance.

**Figure V.12:** Quantitative biodistribution analysis of 1c and 6c in balb/c nu/nu mice bearing subcutaneous SKRC52 tumors. Organ accumulations are reported in units of percent of injected dose per gram of tissue (% ID g\(^{-1}\)). a) 1 h after intravenous administration of 3 nmol 1c (blue) and 6c (red) b) 2 h after intravenous administration of 3 nmol 1c (blue) and 6c (red) c) 4 h after intravenous administration of 3 nmol 1c (blue) and 6c (red) d) Calibration curve (average of triplicates) for the conversion of fluorescence intensity to % ID g\(^{-1}\). Error bars indicate standard deviations. All data points are averages of three mice.

A tumor-to-blood ratio of 13.8:1 was observed 1 h after intravenous injection of 1c (Table V.2) and further improved to 79.2:1 after 4 h. Tumor-to-organ ratios for excretory organs ranged between 0.2:1 for liver and 1.4:1 for kidneys after 1 h (Tables V.1 and 2) but a high level of selectivity was observed for other organs (e.g.,
27.6:1 for tumor to heart after 1 h, Table IV.2). AAZ is a CA ligand with broad isoform selectivity\cite{325}, but the generation of derivatives containing multiple charges may limit their tissue distribution to the extracellular space and thus restrict binding to extracellular membrane-associated CAs. Importantly, tumor targeting was clearly dependent on the CAIX-binding moiety, as revealed by strikingly different areas under the curve of tumor uptake over time (Figure V.11b) and by the fact that the AAZ-based targeted dye conjugate 1c had a 22-fold higher tumor accumulation at 1h than the nontargeted dye 6c (Table V.1). Assuming that 6c is a good model for the tissue distribution of “naked” (i.e., untargeted) anticancer agents, this comparison highlights the potential impact of ligand-based drug delivery of therapeutically relevant doses of drugs into neoplastic masses.

<table>
<thead>
<tr>
<th>Organ</th>
<th>1 h after injection</th>
<th>2 h after injection</th>
<th>4 h after injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(0.7 ± 0.8)</td>
<td>(0.1 ± 0.5)</td>
<td>(ND)</td>
<td>(ND)</td>
</tr>
<tr>
<td>Intestine</td>
<td>1.1</td>
<td>0.0</td>
<td>1.9</td>
</tr>
<tr>
<td>(12.1 ± 2.1)</td>
<td>(34.4 ± 8.1)</td>
<td>(3.8 ± 0.7)</td>
<td>(9.7 ± 12.4)</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.4</td>
<td>0.8</td>
<td>2.6</td>
</tr>
<tr>
<td>(9.7 ± 2.8)</td>
<td>(0.8 ± 0.1)</td>
<td>(2.8 ± 1.8)</td>
<td>(0.6 ± 0.7)</td>
</tr>
<tr>
<td>Liver</td>
<td>0.3</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>(49.6 ± 14.6)</td>
<td>(5.5 ± 1.9)</td>
<td>(17.1 ± 13.2)</td>
<td>(1.5 ± 0.7)</td>
</tr>
<tr>
<td>Lung</td>
<td>1.5</td>
<td>0.3</td>
<td>1.6</td>
</tr>
<tr>
<td>(9.0 ± 3.7)</td>
<td>(1.9 ± 0.3)</td>
<td>(4.6 ± 0.8)</td>
<td>(1.2 ± 1.1)</td>
</tr>
<tr>
<td>Muscle</td>
<td>2.8</td>
<td>2.4</td>
<td>5.0</td>
</tr>
<tr>
<td>(4.7 ± 3.9)</td>
<td>(0.3 ± 0.1)</td>
<td>(1.5 ± 0.8)</td>
<td>(ND)</td>
</tr>
<tr>
<td>Spleen</td>
<td>12.2</td>
<td>ND</td>
<td>18.1</td>
</tr>
<tr>
<td>(1.1 ± 1.0)</td>
<td>(0.0 ± 0.2)</td>
<td>(0.4 ± 0.4)</td>
<td>(ND)</td>
</tr>
<tr>
<td>Tumor</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>(13.4 ± 3.0)</td>
<td>(0.6 ± 0.4)</td>
<td>(7.4 ± 1.2)</td>
<td>(0.2 ± 0.4)</td>
</tr>
</tbody>
</table>

Table V.1: Accumulation of targeted IRDye750 conjugate 1c and untargeted conjugate 6c in different organs 1, 2 or 4 h after intravenous administration of 3 nmol relative to tumor (tumor to organ ratios) based on data in Figure V.12. Average accumulations in units of % ID g\(^{-1}\) and standard deviations are given in brackets. ND stands for “not determined”. Three mice were used per time point.
**Figure V.13**: Repeat of quantitative biodistribution analysis of 1c in balb/c nu/nu mice bearing subcutaneous SKRC52 tumors including stomach and blood values 1, 2 and 4 h after giving 3 nmol of the dye conjugate intravenously. Organ accumulations are reported in units of %ID g⁻¹. Error bars indicate standard deviations. Data shown are averages of three mice.

<table>
<thead>
<tr>
<th>Organ</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>1.0 (11.2 ± 0.4)</td>
<td>1.0 (4.6 ± 1.4)</td>
<td>1.0 (2.6 ± 0.8)</td>
</tr>
<tr>
<td>Liver</td>
<td>0.2 (59.4 ± 30.1)</td>
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<td>0.4 (7.1 ± 4.6)</td>
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<tr>
<td>Kidney</td>
<td>1.1 (10.6 ± 2.1)</td>
<td>1.0 (4.4 ± 1.1)</td>
<td>0.9 (2.8 ± 1.2)</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.2 (51.3 ± 46.8)</td>
<td>0.5 (9.7 ± 2.0)</td>
<td>0.6 (4.5 ± 3.9)</td>
</tr>
<tr>
<td>Lung</td>
<td>1.3 (8.9 ± 7.7)</td>
<td>1.0 (4.7 ± 1.2)</td>
<td>2.5 (1.1 ± 0.4)</td>
</tr>
<tr>
<td>Heart</td>
<td>27.6 (0.4 ± 0.2)</td>
<td>94.9 (0.0 ± 0.2)</td>
<td>45.5 (0.1 ± 0.2)</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.8 (4.0 ± 4.0)</td>
<td>12.0 (0.4 ± 0.2)</td>
<td>98.3 (0.0 ± 0.3)</td>
</tr>
<tr>
<td>Muscle</td>
<td>5.5 (2.0 ± 0.1)</td>
<td>3.7 (1.3 ± 0.6)</td>
<td>10.3 (0.3 ± 0.5)</td>
</tr>
<tr>
<td>Stomach</td>
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<td>1.1 (4.3 ± 1.1)</td>
<td>0.5 (5.3 ± 5.2)</td>
</tr>
<tr>
<td>Blood</td>
<td>13.8 (0.8 ± 0.2)</td>
<td>18.6 (0.2 ± 0.1)</td>
<td>79.2 (0.0 ± 0.2)</td>
</tr>
</tbody>
</table>

**Table V.2**: Accumulation of targeted IRDye750 conjugate 1c in different organs 1, 2 or 4 h after intravenous administration of 3 nmol relative to tumor (tumor to organ ratios) based on data in Figure V.13. Average accumulations in units of %ID g⁻¹ and standard deviations are given in brackets. Three mice were used per timepoint.

To gain an understanding of the microscopic distribution of small ligand-based targeted dye conjugates inside tumors, 1b and 6b were intravenously injected into SKRC52 tumor-bearing mice. Already after 30 min, 1b (Figure V.14) had diffused outside the immediate surrounding of blood vessels stained by perfusion of the animal with Hoechst 33342 shortly before tumor extraction. Non-targeted dye conjugate 6b did not reach the tumor in detectable levels (Figure V.14). As a further indication of targeting selectivity, fluorescence was restricted to CAIX-positive tissues and excretory organs (Figure V.15).
Figure V.14: Microscopic analysis of tumors after intravenous administration of 50 nmol targeted ligand-Alexa546 conjugate 1b or untargeted conjugate 6b lacking the CAIX ligand at different time points (green). Mice were perfused with Hoechst 33342 (blue) 5 min before organ extraction to mark perivascular regions. Although the conjugate has already started penetrating into the tumor after 30 min, staining of the tumor with 1b is initially highest in well-perfused areas. Later, the staining becomes more homogeneous. After 2 h the staining becomes weaker as the conjugate is starting to get washed out of the tumor. Fluorescence due to 6b cannot be detected inside the tumor, which is in accordance with the lack of macroscopic accumulation observed with 6c (Figures V.11 and 12). Scale bars indicate 200 µm.

The promising tumor-targeting performance of AAZ derivatives motivated us to investigate the therapeutic activity of conjugates, comprising a linker cleavable in the extracellular space and a potent cytotoxic payload. We focused on 7a,b and 8a,b with duocarmycin derivatives as the payload and on DM1 conjugates 9a,b, since these cytotoxic drugs are actively being used for the development of anti-cancer antibody-drug conjugates.\[333, 334\] Since CAIX did not internalize efficiently in our hands (Figure V.6), we hypothesised that the disulfide bond would be cleaved by reducing agents in the tumor extracellular space (e.g., glutathione liberated by dying tumor cells). Subsequently, the drug would diffuse into the neighboring neoplastic cells. A similar process has been proposed for non-internalizing antibody-drug conjugates.\[57\]
Figure V.15: Microscopic analysis of organs 1 h after intravenous injection of 50 nmol targeted Alexa546 conjugate 1b (green). Mice were perfused with Hoechst 33342 (blue) 5 min before organ extraction to estimate tumor perfusion and mark perivascular areas. Strong fluorescence due to 1b can be detected inside the tumor and the intestine. The latter probably is due to hepatobiliary excretion of the dye conjugate. The layer of fluorescence in the stomach most likely corresponds to gastric mucosal epithelial cells, which express CAIX under normal conditions. Kidney and liver also show some fluorescence as a result of conjugate excretion through these organs. Scale bars indicate 200 µm.

The synthetic schemes for the preparation of the drug conjugates 7-9 are described in Figure V.16a. For all conjugates, a charged peptide spacer related to a previously described linker was used in order to improve water solubility.\[^{153}\] The targeting ligand was attached using \(\text{Cu}^1\) catalyzed alkyne-azide cycloaddition since direct peptide coupling of the AAZ succinic acid derivative as in 1a consistently failed. For the duocarmycin conjugates 7a,b and 8a,b, the residual carbonate or carbamate linker was designed to self-immolate upon disulfide cleavage, yielding the active drug.\[^{153, 335, 336}\] Conjugates 7b, 8b and 9b lacked AAZ as the targeting ligand and were used as negative controls.
Drug conjugates bound CAIX in vitro with low nanomolar affinities whilst control compounds only interacted weakly ($K_D$ values $> 1 \, \mu m$), as tested by competitive fluorescence polarization against 1a (Figure V.17).\[337\] As expected, the carbonate 7a ($t_{1/2} = 15 \, h$) was less stable in PBS at 37 °C than the carbamate 8a ($t_{1/2} > 24 \, h$). No decomposition was observed for the DM1 conjugate 9a under the same conditions (Figure V.16b). The stability of 7a and 8a was reduced in mouse serum in vitro ($t_{1/2} = 43$ and 61 min respectively, Figure V.18a), but occurred in a time range compatible with the preferential accumulation of the AAZ conjugates at the tumor site (Figure 2b).\[338\] The DM1 conjugate 9a was significantly more stable ($t_{1/2} = 20 \, h$, Figure
V.18b). The conjugates were cytotoxic when tested in an *in vitro* assay (Figures V.19 and 20).

**Figure V.17:** Binding affinity of drug conjugates 7-9 for recombinant CAIX determined by competitive fluorescent polarization against 1a. Targeted conjugates 7a, 8a and 9a retain binding affinity for recombinant CAIX *in vitro* whereas untargeted controls 7b, 8b and 9b do not exhibit strong binding. $K_D$ values are given in brackets ± standard errors of fit. The $K_D$ for 7b is > 1 µM. Data points are averages of three experiments. Error bars give standard deviations.

**Figure V.18:** a) Stability of 7a and 8a in mouse serum at 37 °C as determined by mass spectrometry/mass spectrometry (MS/MS). The calculated half-life is given in brackets. Errors of fit were < 1 min and are thus not explicitly given. b) Stability of 9a in mouse serum at 37 °C determined by high-performance liquid chromatography (HPLC). The calculated half-life is given in brackets. Data points are averages of three experiments. Error bars indicate standard deviations. Error of fit was < 3 min and is thus not explicitly given.
Figure V.19: Toxicity of duocarmycin-derivative conjugates 7 and 8 and the free payload 14 towards CAIX expressing SKRC52 cells. a) Cells were incubated for 72 h in the presence of various concentrations of test compound. b) Cells were incubated with the test compound for 1 h, washed once and then incubated with fresh medium for 72 h. The cell killing EC$_{50}$s are given in brackets ± standard error of fit. Data points are averages of three experiments. Error bars indicate standard deviations. Cytotoxicities of targeted and non-targeted conjugates are comparable in the absence of efficient internalization.

Figure V.20: Toxicity of DM1 conjugates 9a and b, the free thiol drug DM1 and the methylated analog DM1SMe (15) towards CAIX expressing SKRC52 cells. a) Cells were incubated for 72 h in the presence of various concentrations of test compound. b) Cells were incubated with the test compound for 1 h, washed once and then incubated with fresh medium for 72 h. Cell killing EC$_{50}$s are given in brackets ± standard error of fit. Data points are averages of three experiments. Error bars indicate standard deviations. Cytotoxicities of targeted and non-targeted conjugates are comparable in the absence of efficient internalization.

The therapeutic activity of the duocarmycin-derivative and DM1 conjugates 7-9 was tested in mice bearing subcutaneous SKRC52 tumors. Drugs were administered at a recommended dose, which was experimentally determined by dose escalation
studies (Figure V.21). Equimolar amounts of untargeted conjugates 7b, 8b and 9b together with AAZ were used as negative controls.

![Graphs](image)

**Figure V.21:** Estimation of the recommended therapy dose of a) 7a and b) 8a in nude mice at a schedule of five injections on five consecutive days compared to vehicle (5% DMSO in PBS pH 7.4). One mouse was used for each dose. When the animal did not lose more than 5% of its initial body weight over 15 days after the initial injection, it was assumed, that the dose was well tolerated. c) Estimation of the recommended therapy dose and schedule of DM1 conjugate 9a in SKRC52 tumor bearing nude mice. One mouse was used for testing each dosing scheme. Injections were given daily starting on day 0 in 5% DMSO in PBS pH 7.4 (150 µL). Six doses of 60 nmol 9a were tolerated with only minimal weight loss. Since the animals in this study weighed on average 18% less than those used in the therapy study (Figure V.23), a dose of 70 nmol per injection was used for the final experiment. The number of injections was also increased from 6 to 7 on 7 consecutive days.

The therapeutic results obtained with the duocarmycin-AAZ conjugates only indicated a modest tumor growth inhibition effect (Figure V.22a). Nevertheless, targeted carbonate 7a gave rise to statistically significant tumor growth retardation compared to mice that only received vehicle ($p < 0.0001$) and mice receiving untargeted conjugate 7b plus equimolar amounts of AAZ ($p < 0.05$). The carbamate-based
constructs 8a and 8b did not lead to any retardation in tumor growth. It seems reasonable that the low affinity of 8a towards the antigen \( K_D = 40.3 \pm 2.6 \text{ nm} \) versus \( K_D = 7.3 \pm 0.5 \text{ nm} \) for 7a) and inefficient extracellular activation may have been partly responsible for this effect. The treatment could be performed with a weight loss lower than 15% of body weight (Figure V.22b).

![Figure V.22](image.png)

**Figure V.22:** Therapy experiments a) Growth of SKRC52 xenografts in balb/c nu/nu mice treated \( 5 \times \) on 5 consecutive days (arrows) with 4 nmol of duocarmycin derivative-based drug conjugates 7-8, or vehicle (5% DMSO in PBS pH 7.4). Untargeted conjugates 7b and 8b were administered with equimolar amounts of AAZ. Error bars give standard errors. b) Change of average weight of animals treated with compounds from a) Error bars give standard deviations. * indicates \( p < 0.05 \); **** indicates \( p < 0.0001 \). Groups were stopped when the tumor of one animal in the group reached \( > 2,000 \text{ mm}^3 \) in volume or weight dropped by \( > 15\% \). Each group consists of 5 or 6 animals.

For the DM1 conjugate 9a, a potent anti-tumor effect was observed at doses, which gave only minimal toxicity \( (i.e., \) no detectable body weight loss giving \( 7 \times 70 \text{ nmol of DM1-conjugate 9a on 7 consecutive days, Figure V.23)\). During the treatment period tumors shrunk and continued to reduce in volume for 7 additional days. Only 20 days after the start of treatment, tumors started regrowing, as a consequence that mice had not received any additional drug treatment. Importantly, neither sorafenib nor sunitinib, which represent the most commonly used chemotherapeutic agents for the treatment of kidney cancer\(^{[339]}\), exhibited any detectable antitumor effect, in line with previous reports in different models of kidney cancer.\(^{[340]}\) These findings suggest that the targeted delivery of potent cytotoxic agents may provide a therapeutic advantage.
compared to the current standard of care. DM1 may be a particularly suitable payload for the development of targeted cytotoxics, since the presence of e.g., an ester moiety in its structure may facilitate its detoxification in clearance-related organs, thus sparing healthy tissues.\textsuperscript{262, 341}

\textbf{Figure V.23}: Therapy experiments c) Growth of SKRC52 xenografts in balb/c nu/nu mice treated 7× on 7 consecutive days (arrows) with 70 nmol of DM1-based drug conjugates 9a and 9b, or vehicle (5% DMSO in PBS pH 7.4). Untargeted conjugate 9b was administered with equimolar amounts of AAZ. The targeted conjugate 9a has a significantly stronger anti-tumor effect than the untargeted conjugate 9b and leads to prolonged suppression of tumor growth. After 20 days, tumors started regrowing as a consequence that mice had not received any additional drug treatment. Clinically used kinase inhibitors sorafenib and sunitinib were given as further controls at a standard dose of 30 mg/kg but did not exhibit any activity. Error bars give standard errors. d) Change of average weight of animals treated with compounds from c) Error bars give standard deviations. * indicates $p < 0.05$; **** indicates $p < 0.0001$. Groups were stopped when the tumor of one animal in the group reached $> 2,000$ mm$^3$ in volume or weight dropped by $> 15\%$ with the exception of 9a where all animals were still alive after 40 days. Each group consists of 5 or 6 animals.

3. Discussion

To the best of our knowledge, this is the first report of a therapeutic effect of small molecule-drug conjugates directed against CAIX, a validated and accessible marker of renal cell carcinoma. Our work demonstrates that targeting non-internalizing antigens with small molecule conjugates can lead to a preferential product uptake at the tumor site and release of cytotoxic payloads, with a potent anti-tumor activity. A
judicious choice of linker-payload combinations contributes to therapeutic performance, as evidenced by the different results obtained with conjugates of DM1 and duocarmycin derivatives, two widely used cytotoxic drugs for ADC development.\textsuperscript{[333, 334]}

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


1. Introduction

Cytotoxic drugs are routinely used for cancer chemotherapy but typically only reach tumours in minimal quantities. The remaining dose accumulates in excretory organs and other healthy tissues thus causing serious dose-limiting toxicity. Consequently, there is a pressing need for the development of novel chemical strategies for the targeted delivery of highly potent cytotoxic agents into neoplastic lesions.

As one possible solution, monoclonal antibodies specific for cell-surface tumour antigens have been proposed as delivery vehicles. By attaching a highly toxic effector to an antibody through a cleavable linker, an antibody drug conjugate (ADC) is obtained. It selectively carries its payload into the diseased tissue where the drug is released. Healthy organs, on the other hand, are mostly spared.

As an alternative to antibodies, low molecular weight ligands of tumour markers have emerged as attractive targeting agents. Small molecules offer the advantage of deeper tissue penetration, faster pharmacokinetics, lower immunogenicity and more facile accessibility by total organic synthesis than macromolecular conjugates.

While for antibody-based pharmacodelivery applications it is well understood that bivalent antibody formats should be preferred, this is less clear for small molecule conjugates. Some studies with ligands of prostate specific membrane antigen (PSMA), RGD-peptides and bombesin-analogues have claimed superior targeting with bivalent conjugates. Other reports failed to show statistically significant differences in binding of monovalent and bivalent ligands to cells in vitro or in tumour accumulation at late time points in vivo. Moreover, most
small molecule-drug conjugates, which currently are in industrial development, are based on monovalent targeting moieties.$^{[140, 141, 324]}$

In this study we primarily investigated the tumour targeting performance of monovalent and bivalent ligands to carbonic anhydrase IX (CAIX) in SKRC52 renal cell carcinoma xenografts, which constitutively express the antigen as often observed in this type of tumour due to loss-of-function of the von Hippel-Lindau tumour suppressor protein (Figure VI.1).$^{[281]}$ More than 140,000 people die of kidney cancer each year (WHO data) and >80% of renal cell carcinoma are constitutively CAIX-positive$^{[281]}$ making this indication a worthwhile and ideal target for our drug delivery approach. Bivalent binders exhibited a higher tumour uptake than monovalent ones. Furthermore, bivalent ligands coupled to the potent cytotoxic maytansinoid DM1$^{[333]}$ via a disulfide linkage could permanently eradicate SKRC52 tumours in a subset of mice whilst previously described monovalent conjugates had at best led to a retardation of tumour growth.$^{[59]}$

**Figure VI.1:** a) Constitutive expression of CAIX (green) in SKRC52 tumour sections. b) Control staining with an irrelevant antibody against hen egg-white lysozyme. Scale bar indicates 200 µm.

**Figure VI.2:** a) Expression of CAIX (green) in A375 tumour sections. Antigen is located at sites distant to blood vessels (red) consistent with expression in response to hypoxia. b) Control staining with an irrelevant antibody against hen egg-white lysozyme. Scale bar indicates 200 µm.
In many other solid tumours, CAIX is expressed in response to hypoxia.\textsuperscript{[165]} It has been reported that in this setting inhibition of CAIX activity with small molecules can retard tumour growth\textsuperscript{[299]}, inhibit metastasis\textsuperscript{[300]} and cancer stem cell expansion in breast cancer in mice.\textsuperscript{[287]} We thus also tested our targeting ligands in a xenograft model, which preferentially expresses CAIX in tumour areas distant to blood vessels (Figure VI.2) consistent with hypoxia using the A375 melanoma model for this purpose.

CAIX is almost undetectable in normal adult tissues, with the exception of certain structures of the gastrointestinal tract.\textsuperscript{[282, 352]} Other CA isoforms are mostly present intracellularly (such as the highly abundant CAII) but certain subtypes may also be found on cell membranes.\textsuperscript{[297, 298]}

2. Results

2.1. \textit{In Vitro} Characterisation of Targeting Ligands

Monovalent acetazolamide (AAZ) derivative 1 and bivalent AAZ derivative 2 were synthesised using standard Fmoc solid phase peptide chemistry (Figure VI.3a). Binding experiments of monovalent and bivalent AAZ derivatives to CAIX using surface plasmon resonance (SPR, Figure VI.3b) indicated a fast association for both compounds ($k_a = 1.48 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and $k_{a1} = 1.28 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, $k_{a2} = 1.36 \times 10^6 \text{ RU}^{-1}\text{s}^{-1}$ respectively). Whilst monovalent ligand 1 completely dissociated from the CAIX-coated surface within seconds ($k_d = 0.015 \text{ s}^{-1}$, $K_D = 10.5 \text{ nM}$), bivalent compound 2 exhibited no apparent dissociation and could only be removed with harsh acid treatment (Figure VI.3b). Flow cytometry with monovalent and bivalent near infrared dye conjugates 3 and 4 and negative control conjugates 5 and 6 lacking the ligand on CAIX-positive SKRC52 cells and CAIX-negative HEK cells\textsuperscript{[59]} indicated a clear ligand-and receptor-dependent binding to cells (Figures VI.4 and 5). The shift in fluorescence intensity for bivalent conjugate 4 was more pronounced than the one observed for monovalent 3, which is consistent with the results obtained from SPR.
Figure VI.3: a) Structures of monovalent and bivalent ligands and dye-conjugates. b) Surface plasmon resonance characterisation of monovalent ligand 1 (top) and bivalent 2 (bottom).

Figure VI.4: Flow cytometric analysis of binding of a) monovalent IRDye750 conjugate 3 and b) bivalent 4 to CAIX-expressing SKRC52 cells compared with untargeted controls 5 and 6 lacking the targeting ligand.

Figure VI.5: Flow cytometric analysis of binding of IRDye750 conjugates 3-6 to CAIX-negative HEK cells. Cells were incubated with conjugates (30 nM) for 1 h at 4 °C followed by washing and analysis. All histograms are superimposable with untreated cells suggesting the absence of non-specific binding.
2.2. *In Vivo* Investigation of Targeting Performance

Encouraged by these results we tested the ability of near infrared dye conjugates 3 and 4 to localise to SKRC52 xenografts *in vivo*, which constitutively express the antigen throughout the tumour (Figure VI.1). Both dye conjugates strongly accumulated in the tumour, as revealed by whole animal near infrared fluorescence imaging and by analysis of the extracted organs (Figure VI.6). Untargeted controls 5 and 6, on the other hand, did not reach the tumour in detectable amounts (Figure VI.7).

![Diagram](image.png)

*Figure VI.6*: Near infrared images of mice bearing SKRC52 tumours injected with 3 nmol of monovalent IRDye750 conjugate 3 or bivalent IRDye750 conjugate 4. Images were taken 2, 4, 8, 12 and 24 h after injection. After the last time point, animals were sacrificed, organs extracted and imaged individually. Organs from left top to right bottom are Tu = SKRC52 tumour, Li = liver, Ki = kidneys, Lu = lungs, In = Intestine, St = stomach, Hr = heart, Mu = muscle, Sp = spleen.
Figure VI.7: Near infrared images of mice bearing SKRC52 tumours injected intravenously with 3 nmol monovalent IRDye750 conjugate 5 or bivalent IRDye750 conjugate 6. The conjugates were identical to those used in Figure VI.6 but lacked the targeting ligand. Images were taken 2, 4, 8, 12, and 24 h after injection. Animals were sacrificed after the last time point, organs extracted and imaged individually. In the absence of a targeting ligand, the dye conjugate did not accumulate inside the tumour in measurable amounts.

While the initial clearance profile was comparable for both targeted molecules, the bivalent conjugate 4 exhibited a significantly longer residence on the tumour (Figures VI.6 and 8). Twenty-four hours after injection, the integrated fluorescence signal in the tumour from bivalent conjugate 4 was 40%, while the monovalent conjugate 3 had decayed to 14% of its initial value (p = 0.002; unpaired two-sided t-test; Figure VI.8b).
Figure VI.8: a) Change of fluorescence signal from muscle over time in SKRC52 xenograft bearing mice. Regions of interest (ROIs) were placed over the right hind leg muscle of mice in Figure VI.6, integrated to obtain total fluorescence and each expressed relative to the signal from the same animal at 2 h. In the absence of antigen in muscle\textsuperscript{[353]} fluorescence from this tissue was used as a surrogate marker for dye in circulation (blood + interstitium). Decay patterns are comparable suggesting that clearance from circulation is broadly the same for monovalent and bivalent conjugates. Data points are averages of three animals. Error bars give standard deviations. b) Fluorescence emanating from a region of interest (ROI) laid over the SKRC52 tumour relative to the signal from the corresponding ROI at 2 h after injection of 3 nmol monovalent 3 or bivalent dye conjugate 4. Data points are averages of three animals. Error bars give standard deviations. Difference at 24 h is statistically significant ($p = 0.002$; unpaired two-sided t-test).

To gain a better understanding of the absolute tumour uptake of monovalent dye conjugate 3 compared to bivalent 4 and tumour to organ selectivity, organs were extracted, tissues homogenised and fluorescence intensity measured on a per gram basis (Figure VI.9). Comparison to a standard dilution series of IRDye750 in organ homogenate allowed the measurement of absolute uptake levels into organs, as percent injected dose per gram (% ID g\textsuperscript{-1}, Figure VI.10). Bivalent dye conjugate 4 exhibited a >3-fold higher absolute accumulation in tumours compared to monovalent 3 at 24 h (5.3 ± 0.6 versus 1.4 ± 0.6 %IDg\textsuperscript{-1}, Figure VI.9). Compound 4 thus compares very favourable with recently described monoclonal antibodies against CAIX.\textsuperscript{[308]} While uptake into heart, spleen, muscle and circulation in blood relative to tumour was low (tumour:organ > 30), slightly lower tumour to organ ratios were observed for kidneys and stomach for both conjugates. Interestingly, tumour:liver and tumour:intestine ratios were lower for monovalent 3 than for bivalent 4 whilst 4 exhibited a higher tumour:lung ratio than 3 (Tables VI.1 and 2).
Figure VI.9: a) Biodistribution of monovalent IRDye750 conjugate 3 and bivalent IRDye750 conjugate 4 in SKRC52 xenograft bearing balb/c nu/nu mice 24 h after intravenous administration of 3 nmol conjugate. Uptake levels are given as the percentage of the injected dose resident in each organ normalised to organ weight in g (% ID g\(^{-1}\)) and were obtained by comparison of fluorescence from organ homogenate with organ-specific calibration curves (Figure VI.10). b) Total fluorescence normalised to organ weight originating from the same organs as described in the left part of the figure. Fluorescence was determined by integration over near infrared images of intact organs and is given as radiance efficiency in units of (photons s\(^{-1}\) cm\(^{-2}\) sr\(^{-1}\) (µW cm\(^{-2}\)) \times 10^9 normalised to organ weight in g. Values are averages of three experiments. Error bars give standard deviations. A 2-sided unpaired t-test was used to test for statistically significant differences. * p < 0.05, ** p < 0.01, *** p < 0.005, **** p < 0.001, ***** p < 0.0005.

Figure VI.10: Calibration curves for the estimation of IRDye750 uptake in different organs. Organ homogenates were spiked with different amounts of IRDye750 and fluorescence emanating from homogenates was measured on an IVIS imaging system as radiance efficiency in units of (photons s\(^{-1}\) cm\(^{-2}\) sr\(^{-1}\) (µW cm\(^{-2}\)) \times 10^9. With the exception of heart, data points are averages of three organ samples. Error bars give standard deviations. Tumours are SKRC52 xenografts.
Liver   Kidney   Lung   Intestine   Stomach   Heart   Spleen   Muscle   Blood
3     2.0 ± 0.3   2.5 ± 0.4   8.8 ± 1.4   3.4 ± 0.6   2.4 ± 0.4   ND   34 ± 6   285 ± 61   ND
4     16 ± 3     2.8 ± 0.4   2.7 ± 0.4   10 ± 2     3.4 ± 0.5   91 ± 16   29 ± 6   33 ± 5   ND

Table VI.1: Selectivity of uptake of monovalent IRDye750 conjugate 3 and bivalent IRDye750 conjugate 4 into SKRC52 tumours relative to other organs 24 h after intravenous administration of 3 nmol as determined from organ uptake levels in % ID g⁻¹ (Figure VI.9a). Tumour:organ ratios are given as averages of three animals ± standard deviation.

<table>
<thead>
<tr>
<th>Liver</th>
<th>Kidney</th>
<th>Lung</th>
<th>Intestine</th>
<th>Stomach</th>
<th>Heart</th>
<th>Spleen</th>
<th>Muscle</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3.0 ± 0.8</td>
<td>3.5 ± 1.1</td>
<td>5.2 ± 1.0</td>
<td>3.2 ± 0.9</td>
<td>1.9 ± 0.9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>12.8 ± 1.1</td>
<td>2.4 ± 0.2</td>
<td>1.6 ± 0.1</td>
<td>3.7 ± 0.3</td>
<td>1.3 ± 0.4</td>
<td>17.5 ± 3.7</td>
<td>ND</td>
<td>14.2 ± 0.0</td>
</tr>
</tbody>
</table>

Table VI.2: Selectivity of uptake of monovalent IRDye750 conjugate 3 and bivalent IRDye750 conjugate 4 into SKRC52 tumours relative to other organs 24 h after intravenous administration of 3 nmol as determined from total fluorescence signal integrated over organs and normalised to organ weight (Figure VI.9b). Tumour:organ ratios are given as averages of three animals ± standard deviation.

In A375 tumours, on the other hand, which preferentially express CAIX at sites distant to blood vessels (Figure VI.2), neither 3 nor 4 visibly accumulated above background (Figures VI.11 and 12). It was thus decided to focus on SKRC52 renal cell carcinoma for therapy studies.
Figure VI.11: Near infrared images of mice bearing A375 tumours on their lower left back injected with 3 nmol of monovalent IRDye750 conjugate 3 or bivalent IRDye750 conjugate 4. Images were taken 2, 4, 8, 12 and 24 h after injection. After the last time point, animals were sacrificed, organs extracted and imaged individually. Organs from left top to right bottom are Tu = A375 tumour, Li = liver, Ki = kidneys, Lu = lungs, In = Intestine, St = stomach, Hr = heart, Mu = muscle, Sp = spleen. In spite of some CAIX expression at sites distant to blood vessels as evidenced by immunofluorescence, neither of the conjugates accumulates at the tumour site.
Figure VI.12: Near infrared images of mice bearing SKRC52 tumours injected intravenously with 3 nmol monovalent IRDye750 conjugate 5 or bivalent IRDye750 conjugate 6. The conjugates were identical to those used in Figure VI.11 but lacked the targeting ligand. Images were taken 2, 4, 8, 12 and 24 h after injection. Animals were sacrificed after the last time point, organs extracted and imaged individually. In the absence of a targeting ligand, the dye conjugate did not accumulate inside the tumour in measurable amounts.

2.3. Therapeutic Efficacy In Vivo

We coupled the potent cytotoxic drug DM1\[^{333}\] through a disulfide bond to the same bivalent scaffold as in dye conjugate 4 to give drug conjugate 7 (Scheme VI.1). We also prepared 8 lacking the targeting ligand as a negative control. Both the targeted drug 7 and untargeted 8 were equally toxic in vitro (Figure VI.13). If conjugates were efficiently internalised in a receptor-dependent fashion and activated intracellularly, targeted conjugate 7 would be expected to accumulate in CAIX-expressing cells and to be more toxic than untargeted drug 8.\[^{140, 324}\] This does not seem to be the case here. We thus hypothesised that the conjugate would accumulate at the tumour site, where reducing agents (e.g., glutathione released from dying cells) would cleave the disulfide bond in extracellular space and lead to drug release. DM1 would then diffusion into adjacent cells to act on its intracellular target. A similar mechanism had
previously been postulated for the activation of non-internalizing disulfide-linked antibody-drug conjugates.\textsuperscript{[56, 57]}

\begin{center}
\textbf{Scheme VI.1:} Structure of bivalent ligand-drug conjugate 7 and negative control conjugate 8 lacking the targeting ligand.
\end{center}

\begin{center}
\textbf{Figure VI.13:} \textit{In vitro} cytotoxicity of targeted DM1 conjugate 7 and untargeted DM1 conjugate 8 towards CAIX-expressing SKRC52 cells. Cell killing $EC_{50}$ values are given in brackets ± error of fit. a) Cells were exposed to toxin over 72 h. b) Cells were exposed to toxin for 1 h, washed and then incubated for 71 h. Targeted and untargeted conjugates exhibit similar cytotoxicity suggesting that receptor-mediated internalisation followed by intracellular activation of the conjugate is inefficient. If bivalent conjugates were actively internalised by receptor-mediated mechanisms and activated intracellularly, targeted conjugate 7 would be expected to be more toxic than conjugate 8 lacking the targeting ligand. Similar results had been observed with monovalent drug conjugates.\textsuperscript{3} Internalising peptide ligands of CAIX have previously been described\textsuperscript{[330]} suggesting that the observed behaviour is related to the nature of our conjugate rather than a general inability of the antigen to internalise.
A preliminary dose finding study with conjugate 7 was conducted. Even a dose as low as 6 nmol on 8 consecutive days led to substantial tumour shrinkage (Figure VI.15). Five doses of 48 nmol within six days completely eradicated the tumour but showed some toxicity. We finally used a therapeutic schedule of 8 times 35 nmol 7 on 8 consecutive days, which was well-tolerated in SKRC52 tumour bearing mice (Figure VI.16). On the 12th day after the start of treatment, two mice receiving 7 were tumour free and the average tumour volume for all mice had dropped from 200 mm$^3$ initial tumour volume to below 50 mm$^3$. The two mice with complete regression and one from the dose escalation study were tumour free 90 days after start of therapy and were thus considered cured. The remaining tumours regrew. Importantly, control conjugates lacking the targeting ligand or bivalent scaffold 2 without the payload did not have a statistically significant antitumour effect. For comparison, a previously described monovalent disulfide linked AAZ-DM1 conjugate given at the maximum tolerated dose had only given rise to a growth retardation in the same model of renal cell carcinoma.\cite{59} Equimolar amounts of free DM1 could not be given due to unacceptably high toxicity.
Figure VI.15: Dose finding study with targeted bivalent DM1 conjugate 7. a) Tumour growth curves b) Weight change over course of therapy experiment. A single animal was used for each condition. Conjugates were administered daily for the indicated number of days at the given dose in PBS containing 5% DMSO v/v with the exception of 48 nmol which was given on 4 consecutive days followed by 1 day of pause and then the final dose. Doses of 8× 6 nmol, 5× 12 nmol and 8× 24 nmol were well tolerated as indicated by only temporary weight loss < 5%. A dose of (4+1)× 48 nmol led to a delayed toxicity on day 10 (weight loss < 10%) and was thus deemed to be above the maximum tolerated dose. The mouse, which had received 8× 24 nmol, was still tumour free 90 days after start of therapy and was thus deemed cured. The tumours of all other mice regrew after the end of therapy.

Figure VI.16: a) Tumour growth curves of SKRC52 xenograft bearing animals injected with 8x 35 nmol unconjugated ligand 2, targeted conjugate 7, control conjugate 8 or vehicle as control (arrows). Only the targeted drug conjugate brings about a tumour-shrinkage and cures 2 of 6 mice after 12 days. Data represent averages ± standard errors. * indicates statistically significant difference to all other groups with p < 0.05. b) Weight change of animals shown in a). All conjugates were well-tolerated as evidence by the absence of weight loss >5%. Data represent averages ± standard deviations.
3. **Discussion**

In this study we showed that a bivalent AAZ-dye conjugate efficiently accumulated in constitutively CAIX-expressing SKRC52 kidney cancer xenograft tumours and stayed on target longer than the corresponding monovalent conjugate. While tumour:organ selectivities of both monovalent and bivalent conjugates were comparable for most organs, tumour:organ ratios differed for lung, liver and intestine, suggesting that linker chemistry contributes to *in vivo* targeting selectivity. We thus anticipate that an imaging-based *in vivo* evaluation of entire drug conjugates consisting of different combinations of CAIX-ligands including recently described structures, which have so far not been evaluated for drug delivery applications\(^{[354-356]}\), new linkers and drugs\(^{[357]}\) may represent an important step towards clinical translation activities. Ideally, conservative insertion of radioisotopes (*e.g.*, \(^{19}\text{F}\) to \(^{18}\text{F}\)) at judiciously chosen positions may facilitate theranostic investigations, while preserving the chemical identity and pharmacokinetic properties of the candidate compounds.

The therapy experiments revealed that a bivalent small molecule-drug conjugate directed against CAIX cured 2 of 6 mice, bearing subcutaneous SKRC52 human renal cell carcinoma xenografts. The model has previously proved to be resistant to standard-of-care therapies such as sunitinib and sorafenib, which did not lead to any detectable tumour growth retardation even when used at the maximal tolerated dose\(^{[59]}\). Furthermore, monovalent small molecule-DM1 conjugates directed against CAIX had been shown to inhibit tumour growth, but were not able to elicit cures in SKRC52 xenografts\(^{[59]}\). The activity of the drug conjugate 7 was clearly ligand-dependent and the bivalent CAIX ligand alone did not exhibit any antitumour effect supporting the mechanism of targeted drug delivery.

In spite of the fact that CAIX was detectable by immunofluorescence microscopy in A375 tumours in areas distant to blood vessels (Figure VI.2), neither mono- nor bivalent ligand-dye conjugates visibly accumulated at the tumour site suggesting inaccessibility or insufficient expression levels of the antigen for targeting. Counter to this, it has previously been shown that AAZ-dye conjugates could accumulate in HT29 tumours\(^{[358]}\), which express CAIX in a similar pattern to A375\(^{[359]}\). An in depth quantitative analysis of targeting performance, antigen expression, sub-cellular localisation, antigen shedding and vascular permeability amongst other factors will be required to understand this phenomenon. Based on the experimental findings of this
study, a simplistic assumption that a given CAIX ligand should perform equally well in different tumour models does not appear to be justified.

4. **Conclusion**

Bivalent small molecule-drug conjugates directed against CAIX may represent an efficient chemical strategy for the delivery of therapeutic agents into neoplastic masses. Given that targeting agents do not necessarily accumulate in all tumours expressing the antigen, the use of companion diagnostics to quantitatively assess targeting performance in patients prior to therapy will be key for clinical translation.\[165\] Due to the high proportion of cases with constitutive CAIX expression, renal cell carcinoma may be a particularly attractive target indication for our drug delivery approach. Kidney cancer is a sizable and still incurable disease and there is a strong need for the development of better therapeutic agents. The conjugate presented here holds promises for further development in patients with CAIX-overexpressing kidney cancer.
VII. DISCUSSION, CONCLUSION AND OUTLOOK

1. Discussion

We have demonstrated that small molecule ligands, with high binding affinity for the tumor antigen carbonic anhydrase IX (CAIX), can be used as vehicles for the targeted delivery of diverse payloads into antigen-expressing solid tumors. Conjugates of a derivative of the carbonic anhydrase inhibitor acetazolamide (AAZ) with various types of dyes strongly accumulated in CAIX-expressing SKRC52 renal cell carcinoma xenografts in vivo, whereas negative control conjugates lacking the ligand could not be detected inside the tumor in measurable quantities. Disulfide-based conjugates of the potent cytotoxic drug DM1 with the targeting ligand led to a substantial tumor growth retardation in the SKRC52 xenograft model in vivo after intravenous administration. Drug conjugates lacking the targeting ligand did not have a measurable therapeutic effect, compared to saline controls. Importantly, the conjugates were well tolerated as opposed to the free drug, which could not be given at the same dose as the conjugate due to unacceptable toxicities.

In vitro experiments with ligand-dye and drug conjugates suggest that our products, most probably, do not internalize into the target tumor cells, or only do so very inefficiently. Confocal microscopy with ligand-dye conjugates only showed cell surface staining rather than internalization. Flow cytometry experiments indicated some internalization, which, however, could not be sustained over time. Finally, the in vitro cytotoxicity of targeted and untargeted drug conjugates towards CAIX-expressing SKRC52 cells was identical. If substantial amounts of ligand-drug conjugate were internalized and activated intracellularly after binding to CAIX, the targeted conjugate would be expected to be more toxic to antigen-positive cells in vitro compared to negative controls lacking the targeting ligand. A priori this experiment does not tell us whether the uptake or activation step is inefficient (or both). The microscopy and flow cytometry data, however, suggest a lack of efficient internalization. This plays a crucial role for the judicious choice of linker-payload combinations for therapeutic purposes.

It had long been suggested that internalization of ligand-drug conjugates may be an essential requirement for activation. Nevertheless we do see a substantial
therapeutic effect in vivo with our non-internalizing small molecule-drug conjugates. This observation parallels results from previous studies with non-internalising antibody drug conjugates (ADCs) directed against extracellular matrix components of the tumor-associated neovasculature. Disulfide based ADCs, featuring a cemadotin derivative or the maytansinoid DM1 as the payload, could substantially reduce tumor growth or even cure mice depending on the drug being used and the dose.\cite{56, 57} Based on this pronounced activity, it was proposed that reducing agents from dying tumor cells would cleave the disulfide ligand-drug linkage and release the payload. The drug would then enter neighboring cells to exert its therapeutic effect. Further work is required, to unambiguously prove this proposed mechanism of action and understand its efficacy.

By dimerizing the targeting ligand we obtained a delivery vehicle with substantially higher functional binding affinity for the target antigen and the capacity to stay inside SKRC52 tumors longer compared to its monovalent counterparts. Bivalence and avidity effects thus can be important contributors to the targeting efficacy of small molecule drug delivery systems, in full analogy to what has previously been demonstrated for antibody-based tumor targeting strategies.\cite{345, 346} Importantly, as a result of their improved tumor-homing properties, bivalent drug conjugates were shown to lead to durable cures in individual tumor-bearing mice with well-established disease.

In an attempt to explore the wider applicability of our CAIX-directed targeting system, the accumulation of monovalent and bivalent dye conjugates inside A375 melanoma xenografts was investigated. These tumors express CAIX in regions distant to blood vessels suggesting antigen expression in response to hypoxia. Conjugates did not measurably accumulate in the xenografts. Interestingly, it had previously been shown that AAZ-based dye conjugates could accumulate inside HT29 tumors\cite{358}, which express CAIX in a similar pattern as A375 melanoma xenografts.\cite{359} We currently do not fully understand this discrepancy. A detailed investigation of factors affecting targeting beyond antigen expression will be necessary, including tumor vascularization and vascular permeability, subcellular localisation of the antigen and absolute expression levels, to mention only a few.
2. Conclusion
In conclusion, novel small molecule-drug conjugates with potent activity against CAIX-expressing renal cell carcinoma have been developed. In mice bearing SKRC52 renal cell carcinoma xenografts, we observed cures in one third of all animals and substantial tumor growth retardation in all others. Renal cell carcinoma is a still incurable disease with few effective treatment options. We thus believe that our drug delivery strategy deserves further industrial consideration and clinical development programs. Activity in other forms of cancer will be subject to further investigation.

3. Outlook
Work is now underway to further improve the targeting performance of sulfonamide ligands in CAIX-expressing renal cell carcinoma. We are currently pursuing an affinity maturation approach using an encoded self-assembled chemical library (ESAC) approach. One ligand displayed excellent targeting performance in SKRC52 xenografts in vivo and will soon be evaluated for the targeted delivery of DM1 in a therapeutic setting.

So far we have conducted biodistribution studies using fluorescently labeled targeting ligands and homogenized organs for quantification of dye accumulation using near infrared imaging. The gold standard for the execution of quantitative biodistribution studies, however, clearly relies on radioactivity measurements. We are now preparing ligand-chelator conjugates for radio-imaging experiments and biodistribution analysis using radioactive metals and gamma counters. On the one hand, these studies should provide extra confidence about the tumor targeting performance of our molecules. On the other hand, a radio-imaging agent may serve as companion diagnostic, facilitating patient selection and clinical translational activities.

It will be important to systematically study the influence of the drug on the performance of small-molecule cytotoxic conjugates. Different ligand-payload combinations with therapeutic agents, ideally those which have previously been validated in ADC development should be prepared and tested in SKRC52 tumors. Additionally, it would be interesting to study the most promising products in a panel of
CAIX-expressing renal cell carcinoma models, in order to learn more about the robustness of the approach for the treatment of different renal cell carcinoma types. If successful, we believe that the targeting of CAIX-positive tumors with small molecule drug conjugates provides promising candidates for clinical development activities. Safety toxicity studies in primates will be crucially important, before a pilot clinical trial in patients can be considered. As already highlighted above, the development of a companion diagnostic for patient stratification may facilitate product development activities, as recently indicated in the development of folate-based targeted therapeutics.\[165]
VIII. APPENDIX I: MONOVALENT SMALL MOLECULE DRUG CONJUGATES DIRECTED AGAINST CARBONIC ANHYDRASE IX – EXPERIMENTAL PROCEDURES

This section corresponds to the experimental procedures of the journal article entitled “A small-molecule drug conjugate for the treatment of carbonic anhydrase IX expressing tumors” published in Angewandte Chemie in 2014[59] supplemented with additional NMR spectra.

1. Biological Procedures
1.1. Propagation of Errors

During data analysis standard deviations were propagated according to formula (1) as recommended by the National Institute of Standards and Technology.[361]

\[
\sigma_f = \sqrt{\sum_{i=1..n} \left( \frac{\partial f}{\partial x_i} \sigma_i \right)^2}
\]

(1)

Where \( f = f(x_1, x_2, ... x_n) \), \( \sigma_f \) is the standard deviation of function \( f \) and \( \sigma_i \) is the standard deviation of \( x_i \).

1.2. Determination of Ligand K_D by Fluorescence Polarization Measurement

Fluorescently labeled ligands (2 mg) were dissolved in DMSO (100 µL) and diluted 1:2000 into PBS pH 7.4 to determine the stock’s concentration by absorbance measurement at 495 nm (\( \varepsilon_{495} = 72,000 \text{ M}^{-1} \text{ cm}^{-1} \)). Recombinant CAIX was expressed as described previously[308], dialyzed against assay buffer (50 mM tris(hydroxymethyl)aminomethane [TIRS] pH 7.4 containing 1 mM ZnSO_4) at 4 °C overnight and the protein concentration determined by absorbance measurement at 280 nm (\( \varepsilon_{280} = 35,075 \text{ M}^{-1} \text{ cm}^{-1} \)).

In a black 384-well plate in assay buffer (30 µL) fluorescently labeled ligands (5 nM from appropriately diluted DMSO stocks, final DMSO content adjusted to 0.001%) were incubated with increasing concentrations of recombinant carbonic anhydrase IX (4.6 µM to 140 pM in steps of 1:2) for 1 h at room temperature. The fluorescence polarization (FP) was measured on a Spectra Max Paradigm multimode plate reader (Molecular Devices). Experiments were performed in triplicate, mean FP values...
divided by the top-plateau signal and the fractional FP value fit to equation (2) using KaleidaGraph 4.0 (Synergy Software).

\[ FP = \frac{([P]_0 + [L]_0 + K_D) - \sqrt{([P]_0 + [L]_0 + K_D)^2 - 4[P]_0 [L]_0}}{4} \]  

(2)

Where \( FP \) is the fractional fluorescence polarization, \([P]_0\) the total protein concentration, \([L]_0\) the total concentration of the fluorescently labeled ligand and \(K_D\) the dissociation constant in nM.

1.3. **Competitive Fluorescence Polarization Measurement of \( K_D \)**

In a black 384-well plate in assay buffer (see above, 40 \( \mu\)L) fluorescently labeled probe 1a (5 nM from appropriately diluted DMSO stocks) and recombinant carbonic anhydrase IX (25 nM) were incubated with increasing concentrations of unlabeled ligand (2.5 \( \mu\)M to 76 pM in steps of 1:2) for 1 h at room temperature. The FP was measured on a Spectra Max Paradigm multimode plate reader (Molecular Devices). Experiments were performed in triplicate and data analyzed as described by Wang and co-workers.[337]

1.4. **Cell Culture**

SKRC52 and HEK cells were maintained in RPMI medium (Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen) and antibiotic-antimycotic (AA, Invitrogen) at 37 \(^\circ\)C and 5% CO\(_2\). A549 cells were maintained in F-12K medium (Invitrogen) supplemented with 10% FCS (Invitrogen) and AA (Invitrogen) at 37 \(^\circ\)C and 5% CO\(_2\). For passaging, cells were detached using trypsin with ethylenediaminetetraacetic acid (EDTA) 0.05% (Invitrogen) when reaching 90% confluence and re-seeded at a dilution of 1:10.

1.5. **In Vitro Cytotoxicity Assay**

SKRC52 or A549 cells were seeded in 96-well plates in their appropriate culture medium (100 \( \mu\)L) at a density of 5000 cells per well and allowed to grow for 24 h. The medium was replaced with medium containing different concentrations of test substance (100 \( \mu\)L, 300 nM – 15 pM in 1:3 dilution steps) and plates were incubated for 1 h under standard culture conditions. The medium was removed, cells were
gently washed with fresh medium once and new medium was added (100 µL). After 72 h under culture conditions, MTS cell viability dye (20 µL, Promega) was added, the plates were incubated for 1 h 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. EC<sub>50</sub> values were determined by fitting data to the four-parameter logistic equation. Alternatively, the washing step was omitted and cells were incubated for 72 h in the presence of toxic substances.

1.6. Ligand Binding Analysis by Flow Cytometry
Cells were detached from culture plates using a 50 mM EDTA solution in phosphate buffered saline (PBS) pH 7.4, counted and suspended to a final concentration of 1.5 × 10<sup>6</sup> cells mL<sup>-1</sup> in a 1% v/v solution of FCS in PBS pH 7.4. Aliquots of 3 × 10<sup>5</sup> cells (200 µL) were spun down and resuspended in solutions of IRDye750 (Licor) labeled ligands (30 nM) in a 1% v/v solution of FCS in PBS pH 7.4 (200 µL) and incubated on ice for 1 h. Cells were washed once with 200 µL 1% v/v solution of FCS in PBS pH 7.4 (200 µL), spun down, resuspended in a 1% v/v solution of FCS in PBS pH 7.4 (300 µL) containing propidium iodide (1 µg mL<sup>-1</sup>, Invitrogen) and analyzed on a FACS Canto flow cytometer (BD Bioscience). FlowJo Version 8.7 (Treestar) was used for data analysis and visualization.

1.7. Ligand Internalization Analysis by Flow Cytometry
SKRC52 or A549 cells were seeded in 6-well plates in their appropriate culture medium (2 mL) at a density of 1.5 × 10<sup>5</sup> cells per well and allowed to grow for 24 h under culture conditions. The medium was replaced with medium containing IRDye750 (Licor) labeled probes 1c or 6c (2 mL, 30 nM) and plates incubated for 1, 2 or 4 h under standard culture conditions. After washing with PBS pH 7.4 (2 × 2 mL), Trypsin-EDTA 0.05% (500 µL, Invitrogen) was added and plates incubated under standard culture conditions for 15 min. Medium (500 µL) was added, cells pelleted and resuspended in PBS pH 7.4 containing 1% v/v FCS (150 µL). After incubating for 15 min on ice, cells were labeled for 30 min on ice with rabbit anti human CAIX IgG (1:100, Santa Cruz) in PBS pH 7.4 containing 1% FCS (150 µL), washed with PBS
pH 7.4 containing 1% FCS (2 x 150 µL) and labeled for 30 min on ice with goat anti rabbit IgG Alexa488 conjugate (1:100, Invitrogen) in PBS pH 7.4 containing 1% FCS (150 µL). After washing with PBS pH 7.4 containing 1% FCS (2 x 150 µL), cells were pelleted and resuspended in PBS pH 7.4 containing 1% v/v FCS and propidium iodide (300 µL, 1 µg mL⁻¹, Invitrogen) and analyzed on a FACS Canto flow cytometer (BD Bioscience). FlowJo Version 8.7 (Treestar) was used for data analysis and visualization. To inhibit uptake mechanisms, cells were pre-incubated with medium containing 0.2% NaN₃ for 1 h and the NaN₃ concentration maintained throughout the entire experiment. Alternatively, all steps were performed at 0 °C or an excess of AAZ (100 µM) was added to the culture medium.

1.8. Ligand Internalization Analysis by Confocal Microscopy
SKRC52 cells were seeded into 4-well cover slip chamber plates (Sarstedt) at a density of 10⁴ cells per well in RPMI medium (1 mL, Invitrogen) supplemented with 10% FCS, AA and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 10 mM) and allowed to grow for 24 h under standard culture conditions. The medium was replaced with medium containing 1b or 6b (30 nM), after 1 h Hoechst 33342 nuclear dye (Invitrogen) was added and randomly selected colonies image on an Axiovert 200M confocal microscope (Zeiss).

1.9. Stability Determination by Mass Spectrometry/Mass Spectrometry
Targeted carbonate or carbamate 7a or 8a (30 µg) was dissolved in PBS pH 7.4 (1.5 mL) and incubated at 37 °C under gentle agitation. Aliquots (100 µL) were removed at different time points and diluted 1:1 with an internal standard of etodolac (TCI Chemicals) in MeOH (20 µg mL⁻¹). Small molecules were separated from salts using an Oasis WAX online sample preparation column (Waters) on an Alliance HT separation module (50 µL injections, 0.3 mL min⁻¹ 0.1% aq. HCOOH for 3 min followed by 0.3 mL min⁻¹ MeCN for 7 min, Waters) and analyzed by mass spectrometry/mass spectrometry (MS/MS) on a Quattro API spectrometer (Waters) monitoring appropriate multiple reaction monitoring (MRM) transitions for 8a, 8b and etodolac as standard. Measurements were performed in triplicate, peaks integrated and the fraction of intact test compounds calculated as fraction of signal at time t divided by signal at time zero. Since signals due to etodolac were constant over time

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a further correction using the internal standard as reference was omitted. For stability measures in mouse serum, compounds were dissolved in freshly thawed mouse serum (Invitrogen), aliquots taken at different time points and diluted with an equal volume of MeCN. After vigorous vortexting for 1 min, protein precipitate was spun down and the supernatant analyzed as above.

### 1.10. Stability Determination by High-Performance Liquid Chromatography

Targeted DM1 conjugate 9a (230 µg, 140 nmol) was dissolved in PBS pH 7.4 (1 mL) and incubated at 37 °C under gentle agitation. Aliquots (100 µL) were removed at different time points and diluted 1:1 with an internal standard solution of etodolac (TCI Chemicals) in MeCN (20 µg mL⁻¹). Water (600 µL) was added and aliquots of this mixture (50 µL) analyzed over a Syngergi RP Polar column (150 × 4.6 mm, 4 µm, Phenomenex) on an Alliance HT separation module (1 mL min⁻¹ 5% MeCN in 0.1% aqueous TFA to 100% MeCN over 20 min, Waters). Analytes were detected using a Water 2996 photo array UV/VIS detector (Waters). Measurements were performed in triplicate, peaks integrated and the fraction of intact test compounds calculated as fraction of signal at time \( t \) divided by signal at time zero. Since signals due to etodolac were constant over time a further correction using the internal standard as reference was omitted. For stability measures in mouse serum, compounds were dissolved in freshly thawed mouse serum (Invitrogen), aliquots taken at different time points and diluted with an equal volume of MeCN. After vigorous vortexting for 1 min, protein precipitate was spun down and the supernatant analyzed as above.

### 1.11. Animal Studies

All animal experiments were conducted in accordance with Swiss animal welfare laws and regulations under the license number 42/2012 granted by Veterinaeramt des Kanton Zurich.

### 1.12. Implantation of Subcutaneous SKRC52 Tumors

SKRC52 cells were grown to 80% confluence and detached with Trypsin-EDTA 0.05% (Invitrogen). Cells were washed with PBS pH 7.4 once, counted and resuspended in PBS to a final concentration of 6.7 × 10⁷ cells mL⁻¹. Athymic balb/c nu/nu mice, 8-10 weeks of age (Charles River) were anesthetized with isofluorane
and aliquots of $1 \times 10^7$ cells (150 µL of suspension) injected subcutaneously into their lower back.

1.13. IVIS Imaging
Mice bearing subcutaneous SKRC52 tumors (200 – 300 mm$^3$ in size) were injected intravenously with IRDye750 (Licor) labeled CAIX ligands 1c–6c (up to 10 nmol) dissolved in 5% v/v DMSO in PBS pH 7.4 (150 µL). Mice were anesthetized with isoflurane and fluorescence images acquired on an IVIS Spectrum imaging system (Xenogen, exposure 1s, binning factor 8, excitation at 745 nm, emission filter at 800 nm, f number 2, field of view 13.1). Images were taken after 1 h, 2 h, 4 h, 8 h and 12 h and 24 h. Food and water was given ad libitum during that period. Mice were subsequently sacrificed by cervical dislocation. Heart, lung, kidney, liver, spleen, a section of the intestine (100 - 150 mg), skeletal muscle (100 – 150 mg) and the tumor were extracted and imaged individually using above parameters.

1.14. Biodistribution Analysis
Mice (groups of 3 per time point and compound) bearing subcutaneous SKRC52 tumors (200 – 300 mm$^3$ in size) were injected intravenously with IRDye750 (Licor) labeled probes 1c or 6c (3 nmol) dissolved in 5% v/v DMSO in PBS pH 7.4 (150 µL). After 1 h, 2 h or 4 h animals were sacrificed, organs extracted as above, cut into small pieces, weighed and suspended in 1:1 w/v organ homogenization buffer containing EDTA (40 mM), proteinase K (6 mg/ml), Triton X-100 (1.6 µl/ml) and trace amounts of DNase 1 in PBS pH 7.4 (100 µL per 100 mg of tissue). The suspension was homogenized on a TissueLyser organ homogenizer (Quiagen, 25 Hz, 10 min), incubated for 2 h at room temperature and 100 µL of the homogenate transferred to a black 96-well plate. A standard dilution series of 1c in homogenization buffer (750 nM – 47 nM, 25 – 1.5 % ID g$^{-1}$ in steps of 1:2) was spotted alongside the organ samples in triplicate. Fluorescent images of plates were recorded on an IVIS Spectrum imaging system (Xenogen, parameters as above) and analyzed using Living Image software version 4.3.1 (Caliper Life Science) using the built-in region of interest (ROI) tools. Dye concentrations in organ samples in % of injected dose per gram of tissue (% ID g$^{-1}$) were inferred from fluorescence intensities originating from the corresponding well by comparison with the standard dilution series.
1.15. Analysis of Tumor Penetration
Mice bearing subcutaneous SKRC52 tumors (200 – 300 mm$^3$ in size) were injected intravenously with Alexa546 (Invitrogen) labeled probes 1b or 6b (50 nmol) dissolved in PBS pH 7.4 (150 µL). After 1 h, 2 h or 4 h animals were injected with a solution of Hoechst 33342 (Invitrogen, 5.4 mM) in saline (150 µL) and sacrificed after 5 min. Organs were extracted as above and flash-frozen in Neg-50 cryo medium (Thermo Scientific) using liquid nitrogen. After warming to -20 °C, samples were cut into sections of 10 µm width and directly imaged on an Axioskop 2 fluorescence microscope (Zeiss).

1.16. Therapy Experiments
SKRC52 xenograft tumors were implanted into balb/c nu/nu mice (Charles River) as described above. After 14 days, mice were randomly assigned into therapy groups of 5 or 6 animals and treatment started. 5 doses of 4 nmol 7a,b, 8a,b or 7 doses of 70 nmol 9a,b each in PBS pH 7.4 (150 µL) containing 5% DMSO were given on 5 or 7 consecutive days and one group was treated with vehicle (5% DMSO in PBS pH 7.4). In the case of 7-9b an equimolar amount of AAZ was added to the injection solution to control for a possible antitumor activity of CAIX inhibitors. Sorafenib and sunitnib were administered at a standard dose of 30 mg/kg as described previously.[340] Animals were weighed and tumor sizes measured daily and the tumor volume calculated according to the formula (long side) × (short side)$^2$ × 0.5. Animals were sacrificed when the body weight fell by more than 15% relative to the first therapy day or when tumors reached a volume of >2000 mm$^3$. Prism 6 (GraphPad Software) was used for data analysis (regular two-way ANOVA with the Bonferroni test).

2. Chemical Procedures
2.1. General Chemical Procedures
Proton ($^1$H) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV400 (400 MHz) or a Bruker AVIII500 (500 MHz) spectrometer. Carbon ($^{13}$C) NMR spectra were recorded on a Bruker AV400 (100 MHz) spectrometer or on a Bruker AVIII500 (125 MHz) spectrometer. Chemical 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 were recorded on a Bruker Daltronics maXis ESI-QTOF mass spectrometer. Calculated and exact m/z values are reported in Daltons.

Analytical and preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) were performed on an Waters Alliance HT RP-HPLC with PDA UV detector, using a Synergi 4µm, Polar-RP 150 × 10 mm 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).

Anhydrous solvents for reactions were purchased from Acros or Fluka. Peptide grade dimethyl formamide (DMF) for solid phase synthesis was bought from ABCR. All other solvents were used as supplied by Fisher Chemicals, Merck or Aldrich in HPLC or analytical grade. IRDye750 N-hydroxysuccinimidyl (NHS) ester was purchased from Licor, Alexa546 NHS ester from Invitrogen, N-Boc protected (S)-1-chloromethyl-6-hydroxy-1,2-dihydrobeno[e]indole (seco CBI) from Anthem Bioscience. DM1 was purchased from Concortis Biosystems. All other reagents were purchased from 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. Brine refers to a saturated solution of sodium chloride. Silica for flash column chromatography was purchased from Sigma.

2.2. Preparation of Previously Described Compounds
Compounds 6c and 11 - 17 were prepared according to previously described methods (Table IV.3).
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*Table IV.3:* Compounds prepared according to previously described methods.
2.3. Chemical Synthesis of New Compounds

*N1-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-N4-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)succinamide fluorescein conjugate – 1a*

25 (7.0 mg, 17 µmol) and fluoresceinisothiocyanate (FITC, 6.7 mg, 17 µmol) were dissolved in dimethylformamide (DMF, 1 mL) and diisopropylethylamine (DIPEA, 8 µL, 48 µmol) was added. The reaction was stirred for 2 h at room temperature, diluted with MeOH (1 mL) and purified over reversed-phase HPLC (80% A / 20% B to 20% A / 80% B over 20 min). Fractions containing the desired product by mass spectrometry (MS) were pooled and lyophilized to give the product as a yellow powder (12 mg, 16 µmol, 95%).

$^1$H-NMR (400 MHz, MeOD-$d_4$) $\delta$ [ppm] = 8.31 (d, $J = 1.5$ Hz, 1H), 7.91 (d, $J = 7.5$ Hz, 1H), 7.25 (d, $J = 8.3$ Hz, 1H), 7.02 (d, $J = 8.4$ Hz, 2H), 6.92 (s, 2H), 6.79 (d, $J = 8.7$ Hz, 2H), 3.85 (br, 2H), 3.75 (t, $J = 5.2$ Hz, 2H), 3.72-3.65 (m, 4H), 3.58 (t, $J = 5.4$ Hz, 2H), 3.38 (t, $J = 5.4$ Hz, 2H), 2.85 (t, $J = 6.4$ Hz, 2H), 2.66 (t, $J = 6.4$ Hz, 2H); $^{13}$C-NMR (125 MHz, DMSO-$d_6$) $\delta$ [ppm] = 180.8, 172.3, 172.2, 171.3, 171.2, 169.0, 164.6, 161.4, 159.9, 152.4, 147.6, 141.8, 129.5, 127.0, 124.5, 116.7, 113.0, 110.3, 102.7, 70.1, 69.6, 68.9, 44.1, 39.0, 30.7, 29.8, signals from PEG linker predicted to overlap; HRMS: (m/z) [M + H]$^+$ calcd. for C$_{33}$H$_{34}$N$_7$O$_{11}$S$_3$, 800.1473; found 800.1470.
N1-(2-(2-aminoethoxy)ethoxy)ethyl)-N4-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)succinamide Alexa546 conjugate – 1b

To 25 (212 µg, 517 nmol) in DMF (2.1 µL) was added Alexa546 NHS ester (100 µg, 86 nmol). DIPEA (2 µL, 12 µmol) and DMF (50 µL) were added and the mixture stirred for 2 h at room temperature. The reaction was diluted with MeOH (50 µL) and purified over reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). Fractions containing the product as identified through its characteristic UV/VIS spectrum (λ<sub>max</sub> = 550 nm) were pooled, lyophilized and dissolved in 100 µL PBS pH 7.4 to give a dark purple solution. Its concentration and the reaction yield were determined by measuring the absorbance at 556 nm (ε<sub>556</sub> = 112,000 M<sup>-1</sup> cm<sup>-1</sup>) of stock samples diluted 1:100 into PBS pH 7.4 (443 µM, 44 nmol, 51%).

HRMS: (m/z) [M + H]<sup>+</sup> calcd. for C<sub>52</sub>H<sub>65</sub>Cl<sub>3</sub>N<sub>9</sub>O<sub>17</sub>S<sub>5</sub>, 1352.2162; found 1352.2157.

**Figure VIII.1:** Analytical HPLC trace of 1b using a Synergi 4µm, Polar-RP 150 x10 mm column at a flow rate of 4 mL/min with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 550 nm.
To 25 (131 µg, 320 nmol) in DMSO (13 µL) was added IRDye750 NHS ester (194 µg, 163 nmol) in DMSO (25 µL) followed by DMF (100 µL) and DIPEA (10 µL, 60 µmol). The solution was stirred for 6 h at room temperature and then directly purified over reversed-phase HPLC (95% A / 5% B to 40% A / 60% B over 30 min). Fractions containing dye conjugate were identified through their characteristic UV/VIS spectrum ($\lambda_{\text{max}} = 750$ nm), pooled, lyophilized and dissolved in dimethylsulfoxide (DMSO, 100 µL) to give a dark green stock solution. Its concentration and the reaction yield were determined by measuring the absorbance at 750 nm ($\varepsilon_{750} = 260,000$ M$^{-1}$ cm$^{-1}$) of stock samples diluted 1:200 into PBS pH 7.4 (1.02 mM, 102 nmol, 63%).

HRMS: (m/z) [M + Na]$^+$ cacld. for C$_{61}$H$_{77}$N$_8$NaO$_{19}$S$_6$, 720.1769; found 720.1760.

Figure VIII.2: Analytical HPLC trace of 1c using a Synergi 4µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL/min with linear gradients of 95% A / 5% B to 40% A / 60% B over 30 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 750 nm.
(S)-N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-3-methyl-2-(4-(4-sulfamoylphenyl)-1H-1,2,3-triazol-1-yl)butanamide fluorescein conjugate – 2a

26 (20 mg, 36 µmol) was dissolved in a mixture of dichloromethane (DCM, 0.5 mL) and TFA (0.5 mL) and stirred for 1 h at room temperature. The solvents were removed under reduced pressure and the residue dissolved in DMF (0.5 mL). DIPEA (31 µL, 187 µmol) was added followed by FITC (14 mg, 36 µmol). The reaction was stirred for 2 h at room temperature, diluted with MeOH (0.5 mL) and purified over reversed-phase HPLC (80% A / 20% B to 20% A / 80% B over 20 min). Fractions containing product by MS were pooled and lyophilized to give the product as a bright yellow powder (18 mg, 23 µmol, 64%).

$^1$H-NMR (500 MHz, DMSO-d$_6$) $\delta$ [ppm] = 10.07 (br s, 1H), 8.85 (s, 1H), 8.74 (t, $J = 5.4$ Hz, 1H), 8.31 (s, 1H), 8.10 (d, $J = 6.8$ Hz, 3H), 7.93 (d, $J = 6.8$ Hz, 2H), 7.75 (d, $J = 5.8$ Hz, 1H), 7.38 (s, 2H), 7.18 (d, $J = 8.5$ Hz, 1H), 6.66-6.54 (m, 6H), 5.07 (d, $J = 8.3$ Hz, 1H), 3.68 (br s, 2H), 3.60-3.56 (m, 6H), 3.48 (t, $J = 5.6$ Hz, 2H), 3.41-3.19 (m, 2H), 2.49-2.45 (m, 1H), 1.01 (d, $J = 6.6$ Hz, 3H), 0.73 (d, $J = 6.6$ Hz, 3H); $^{13}$C-NMR (125 MHz, DMSO-d$_6$) $\delta$ [ppm] = 181.1, 169.0, 167.9, 160.2, 159.1, 158.8, 152.5, 147.3, 145.7, 143.6, 141.8, 134.3, 129.5, 126.8, 125.8, 124.6, 121.9, 117.0, 114.5, 113.2, 110.3, 102.7, 70.1, 70, 69.6, 69.2, 68.9, 44.2, 39.26, 31.6, 19.2, 19.1; HRMS: (m/z) [M + H]$^+$ calcd. for C$_{40}$H$_{42}$N$_7$O$_{10}$S$_2$, 844.2429; found 844.2430.
(S)-N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-3-methyl-2-(4-(4-sulfamoylphenyl)-1H-1,2,3-triazol-1-yl)butanamide IRDye750 conjugate – 2c

26 (178 µg, 321 nmol) in DMSO (34 µL) was added to a mixture of TFA (100 µL) and DCM (100 µL). The reaction was stirred for 1 h at room temperature and the solvent removed under reduced pressure. To the residual solution was added IRDye750 NHS ester (194 µg, 163 nmol) in DMSO (25 µL) followed by DMF (100 µL) and DIPEA (10 µL, 60 µmol). The solution was stirred for 6 h at room temperature and then directly purified over reversed-phase HPLC (95% A / 5% B to 40% A / 60% B over 30 min). Fractions containing dye conjugate were identified through their characteristic UV/VIS spectrum (λ_{max} = 750 nm), pooled, lyophilized and dissolved in DMSO (100 µL) to give a dark green stock solution. Its concentration and the reaction yield were determined by measuring the absorbance at 750 nm (ε_{750} = 260,000 M^{-1} cm^{-1}) of stock samples diluted 1:200 into PBS pH 7.4 (662 µM, 66 nmol, 40%).

HRMS: (m/z) [M + Na]^{2+} calcd. for C_{68}H_{85}N_{8}NaO_{18}S_{5}, 742.2247; found 742.2233.

Figure VIII.3: Analytical HPLC trace of 2c using a Synergi 4µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL/min with linear gradients of 95% A / 5% B to 40% A / 60% B over 30 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 750 nm.
28 (7.5 mg, 14 µmol) was dissolved in a mixture of DCM (1 mL) and TFA (1 mL) and stirred for 30 min at room temperature. The solvent was removed under reduced pressure and the residue dissolved in DMF (1 mL). FITC (5.4 mg, 14 µmol) was added followed by DIPEA (23 µL, 139 µmol) and the reaction stirred for 3 h at room temperature. MeOH (1 mL) was added and the crude reaction mixture purified over reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). Fractions containing the desired product by MS were pooled and lyophilized to yield the title compound as a bright yellow powder (8.1 mg, 11 µmol, 76%).

\[ ^1H-NMR \text{(400 MHz, DMSO-d}_6, \text{two rotamers)} \delta [ppm] = 10.31 (\text{br s}, 1H), 10.23 (\text{br s}, 1H), 9.96 (\text{br s}, 2H), 8.21 (\text{br s}, 2H), 8.02 (\text{br s}, 1H), 7.86-7.77 (\text{m}, 2H), 7.69-7.64 (\text{m}, 3H), 7.16 (\text{m}, 2H), 7.10 (d, J = 8.3 Hz, 1H), 6.61-6.48 (\text{m}, 6H), 3.61-3.38 (\text{m}, 12H); \]

\[ ^{13}C-NMR \text{(125 MHz, DMSO-d}_6, \text{two rotamers, signals of PEG linker predicted to overlap)} \delta [ppm] = 181.1, 169.0, 168.5, 166.0, 160.2, 159.3, 159.0, 157.3, 152.4, 142.5, 141.9, 141.0, 139.6, 138.3, 129.5, 127.2, 127.0, 126.9, 124.5, 122.61, 120.0, 113.1, 110.5, 102.7, 70.1, 70.0, 69.1, 68.5, 68.9, 49.1, 44.1; \]

HRMS: (m/z) [M + H]^+ calcd. for C\text{30}H\text{34}ClN\text{8}O\text{9}S\text{2}, 821.1573; found 821.8567.
28 (174 µg, 328 nmol) in DMSO (19 µL) was added to a mixture of TFA (100 µL) and 
DCM (100 µL). The reaction was stirred for 1 h at room temperature and the volatile 
solvents removed under reduced pressure. To the residual solution, IRDye750 NHS 
ester (194 µg, 163 nmol) in DMSO (25 µL) was added followed by DMF (100 µL) 
and DIPEA (10 µL, 60 µmol). The solution was stirred for 6 h at room temperature 
and then directly purified over reversed-phase HPLC (95% A / 5% B to 40% A / 60% 
B over 30 min). Fractions containing dye conjugate were identified through their 
characteristic UV/VIS spectrum (λ<sub>max</sub> = 750 nm), pooled, lyophilized and dissolved in 
DMSO (100 µL) to give a dark green stock solution. Its concentration and the 
reaction yield were determined by measuring the absorbance at 750 nm (ε<sub>750</sub> = 
260,000 M<sup>-1</sup> cm<sup>-1</sup>) of stock samples diluted 1:100 into PBS pH 7.4 (510 µM, 51 nmol, 
31%).

HRMS: (m/z) [M]<sup>3-</sup> calcd. for C<sub>64</sub>H<sub>77</sub>ClN<sub>9</sub>O<sub>17</sub>S<sub>5</sub>, 479.4582; found 479.4569.

**Figure VIII.4:** Analytical HPLC trace of 3c using a Synergi 4µm, Polar-RP 150 × 10 mm column at a 
flow rate of 4 mL/min with linear gradients of 95% A / 5% B to 40% A / 60% B over 30 min (A = 
Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 750 nm. Different gradients and 
using a waters XTerra 5 µm 150 × 10 mm C18 column did not give better peak shapes.

**(E)-N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-3-((4-((4-
sulfamoylphenyl)diazenyl)phenyl)amino)propanamide IRDye750 conjugate – 4c**

![Chemical structure of 4c]
29 (188 µg, 320 nmol) in DMSO (20 µL) was added to a mixture of TFA (100 µL) and DCM (100 µL). The reaction was stirred for 1 h at room temperature and the volatile solvents removed under reduced pressure. To the residue, IRDye750 NHS ester (194 µg, 163 nmol) in DMSO (25 µL) was added followed by DMF (100 µL) and DIPEA (10 µL, 60 µmol). The solution was stirred for 6 h at room temperature and then directly purified over reversed-phase HPLC (95% A / 5% B to 40% A / 60% B over 30 min). Fractions containing dye conjugate were identified through their characteristic UV/VIS spectrum (λ_max = 750 nm), pooled, lyophilized and dissolved in DMSO (100 µL) to give a dark green stock solution. Its concentration and the reaction yield were determined by measuring the absorbance at 750 nm (ε_{750} = 260,000 M^{-1} cm^{-1}) of stock samples diluted 1:100 into PBS pH 7.4 (390 µM, 39 nmol, 24%).

HRMS: (m/z) [M + Na]^{2+} calcd. for C_{70}H_{85}N_8NaO_{18}S_5, 754.2247; found 754.2248.

![Figure VIII.5: Analytical HPLC trace of 4c using a Synergi 4µm, Polar-RP 150 x 10 mm column at a flow rate of 4 mL/min with linear gradients of 95% A / 5% B to 0% A / 100% B over 30 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 750 nm.](image)

N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-4-sulfamoylbenzamide fluorescein conjugate – 5a

31 (5.0 mg, 12 µmol) was dissolved in a mixture of DCM (0.5 mL) and TFA (0.5 mL) and stirred for 1 h at room temperature. The solvents were removed under reduced pressure and the residue dissolved in DMF (0.5 mL). DIPEA (31 µL, 187 µmol) was added followed by FITC (4.5 mg, 12 µmol). The reaction was stirred for 2 h at room
temperature, diluted with MeOH (0.5 mL) and purified over reversed-phase HPLC (80% A / 20% B to 20% A / 80% B over 20 min). Fractions containing product by MS were pooled and lyophilized to give the product as a bright yellow powder (6.3 mg, 10 µmol, 83%).

$^1$H-NMR (500 MHz, DMSO-d$_6$) δ [ppm] = 8.74 (t, $J = 5.6$ Hz, 1H), 8.30 (s, 1H), 8.14 (s, 1H), 8.00 (d, $J = 8.4$ Hz, 2H), 7.90 (d, $J = 8.4$ Hz, 2H), 7.74 (d, $J = 7.6$ Hz, 1H), 7.48 (s, 2H), 7.18 (d, $J = 8.3$ Hz, 1H), 6.65-6.54 (m, 7H), 3.68 (br s, 2H), 3.61-3.56 (m, 8H), 3.47-3.42 (m, 2H); $^{13}$C-NMR (125 MHz, DMSO-d$_6$, signals from PEG linker predicted to overlap) δ [ppm] = 181.1, 169.0, 165.8, 160.0, 159.1, 158.8, 152.3, 147.5, 146.7, 141.8, 137.8, 129.5, 128.3, 127.0, 126.1, 124.6, 116.9, 113.1, 110.2, 102.7, 70.1, 70.0, 69.3, 68.9, 44.2; HRMS: (m/z) [M + H]$^+$ calcd. for C$_{34}$H$_{33}$N$_4$O$_{10}$S$_2$, 721.1633; found 720.1620.

$N$-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-4-sulfamoylbenzamide IRDye750 conjugate – 5c

31 (138 µg, 320 nmol) in DMSO (29 µL) was added to a mixture of TFA (100 µL) and DCM (100 µL). The reaction was stirred for 1 h at room temperature and the volatile solvents removed under reduced pressure. To the residual solution, IRDye750 NHS ester (194 µg, 163 nmol) in DMSO (25 µL) was added followed by DMF (100 µL) and DIPEA (10 µL, 60 µmol). The solution was stirred for 6 h at room temperature and then directly purified over reversed-phase HPLC (95% A / 5% B to 40% A / 60% B over 30 min). Fractions containing dye conjugate were identified through their characteristic UV/VIS spectrum ($\lambda_{\text{max}} = 750$ nm), pooled, lyophilized and dissolved in DMSO (100 µL) to give a dark green stock solution. Its concentration and the reaction yield were determined by measuring the absorbance at 750 nm ($\varepsilon_{750} = 260,000$ M$^{-1}$ cm$^{-1}$) of stock samples diluted 1:200 into PBS pH 7.4 (1.23 mM, 123 nmol, 75%).
HRMS: (m/z) [M + 2H]\(^+\) calcd. for C\(_{62}\)H\(_{78}\)N\(_{5}\)O\(_{18}\)S\(_{5}\), 1340.3951; found 1340.3932.

**Figure VIII.6:** Analytical HPLC trace of 5c using a Synergi 4µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL/min with linear gradients of 95% A / 5% B to 40% A / 60% B over 30 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 750 nm.

tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate fluorescein conjugate – 6a

19 (10 mg, 40 µmol) and FITC (16 mg, 41 µmol) were dissolved in DMF (1 mL) and DIPEA (10 µL, 48 µmol) was added. The reaction was stirred for 2 h at room temperature, diluted with MeOH (1 mL) and purified over reversed-phase HPLC (80% A / 20% B to 20% A / 80% B over 20 min). Fractions containing the desired product by MS were pooled and lyophilized to give the product as a yellow powder (18 mg, 29 µmol, 72%).

\(^1\)H-NMR (500 MHz, DMSO-d\(_6\)) \(\delta\) [ppm] = 8.34 (br, 1H), 7.76 (br, 1H), 7.18 (d, \(J = 8.3\) Hz, 1H), 6.71-6-69 (m, 2H), 6.37-6.56 (m, 6H), 3.63-3.53 (m, 8H), 3.39 (t, \(J = 6.0\), 2H), 3.07 (br, 2H), 1.35 (s, 9H); \(^{13}\)C-NMR (125 MHz, DMSO-d\(_6\)) \(\delta\) [ppm] = 180.9, 169.0, 159.9, 156.0, 152.4, 147.4, 141.8, 129.5, 129.7, 127.0, 124.5, 116.7, 113.0, 110.3, 110.2, 102.7, 78.1, 70.1, 70.0, 69.7, 68.9, 67.02, 44.0, 28.6; HRMS: (m/z) [M + H]\(^+\) calcd. for C\(_{32}\)H\(_{36}\)N\(_3\)O\(_9\)S, 638.2167; found 638.2160.
** tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate Alexa546 conjugate – 6b 

To **19** (233 µg, 943 nmol) in DMSO (6 µL) was added Alexa546 NHS ester (100 µg, 86 nmol). DIPEA (2 µL, 12 µmol) and DMF (50 µL) were added and the mixture stirred for 2 h at room temperature. The reaction was diluted with MeOH (50 µL) and purified over reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). Fractions containing the product as identified through its characteristic UV/VIS spectrum were pooled (λ_{max} = 550 nm), lyophilized and dissolved in 100 µL PBS pH 7.4 to give a dark purple solution. Its concentration and the reaction yield were determined by measuring the absorbance at 556 nm (ε_{556} = 112,000 M^{-1} cm^{-1}) of stock samples diluted 1:100 into PBS pH 7.4 (555 µM, 56 nmol, 65%).

HRMS: (m/z) [M + 2H]^+ calcd. for C_{51}H_{67}Cl_{3}N_{5}O_{15}S_{3}, 1190.2856; found 1190.2859.

**Figure VIII.7:** Analytical HPLC trace of **5b** using a Synergi 4µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL/min with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 550 nm.
AAZ targeted charged linker 11a (7.8 mg, 8.6 µmol) and 12 (5.1 mg, 7.2 µmol) were dissolved in degassed MeOH (0.5 mL) and stirred for 6 h at room temperature. The reaction mixture was directly purified over reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min), fractions containing the product by MS were pooled and lyophilized to give the title compound as a white powder (5.5 mg, 3.7 µmol, 47%).

$^1$H-NMR (500 MHz, DMSO-d$_6$) δ [ppm] = 11.57 (s, 1H), 8.36 (s, 1H), 8.31 (br s, 3H), 8.20 (d, $J$ = 7.5 Hz, 1H), 8.06 (d, $J$ = 8.5 Hz, 1H), 8.03 (br s, 2H), 7.89 (d, $J$ = 8.5 Hz, 2H), 7.85 (s, 1H), 7.63 (t, $J$ = 8.0, 1H), 7.53 (t, $J$ = 8.0 Hz, 1H), 7.31 (s, 1H), 7.21 (br s, 4H), 7.13 (d, $J$ = 2.0 Hz, 1H), 7.02 (s, 1H), 4.87 (t, $J$ = 10.0 Hz, 1H), 4.63-4.52 (m, 5H), 4.45-4.40 (m, 1H), 4.29-4.26 (m, 6H), 4.10 (dd, $J$ = 11.2, 3.1 Hz, 1H), 4.00 (dd, $J$ = 11.2, 6.9 Hz, 1H), 3.84 (s, 3H), 3.51-3.49 (m, 2H), 3.25-3.21 (m, 1H), 3.14-3.11 (m, 2H), 3.07-3.02 (m, 3H), 2.90 (s, 6H), 2.73-2.57 (m, 6H), 2.55-2.45 (m, 2H), 2.13 (t, $J$ = 7.1 Hz, 2H), 1.96-1.90 (m, 2H), 1.81-1.73 (m, 3H), 1.55-1.40 (m, 5H); HRMS: (m/z) [M + 2H]$^{2+}$ calcd. for C$_{60}$H$_{78}$ClN$_{17}$O$_{19}$S$_{4}$, 751.7110; found 751.7109.

**Figure VIII.8:** Analytical HPLC trace of 7a using a Synergi 4µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL/min with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 260 nm.
Untargeted CBI Carbonate – 7b

Activated carbonate 12 (5.0 mg, 7.1 µmol) and untargeted charged linker 11b (10 mg, 14 µmol) were dissolved in degassed MeOH (0.5 mL) and stirred for 6 h at room temperature. The reaction mixture was directly purified over reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min), fractions containing the product by MS were pooled and lyophilized to give the title compound as a white powder (4.1 mg, 3.1 µmol, 43%).

1H-NMR (500 MHz, DMSO-d6) δ [ppm] = 12.30 (br, 3H), 11.57 (s, 1H), 8.37 (s, 1H), 8.21 (d, J = 7.4 Hz, 1H), 8.17 (br s, 2H), 8.06 (d, J = 8.5 Hz, 1H), 7.96 (d, J = 7.3 Hz, 1H), 7.89 (d, J = 8.5 Hz, 1H), 7.82 (s, 1H), 7.64 (t, J = 7.5 Hz, 1H), 7.53 (t, J = 8.0 Hz, 1H), 7.40-6.77 (br, 4H), 7.31 (s, 1H), 7.13 (d, J = 1.9 Hz, 1H), 7.03 (s, 1H), 4.86 (t, J = 10.1 Hz, 1H), 4.63-4.41 (m, 7H), 4.30-4.21 (m, 5H), 4.10 (dd, J = 11.1, 2.8 Hz, 1H), 4.00 (dd, J = 11.2, 7.0 Hz, 1H), 3.85 (s, 3H), 3.54-3.52 (m, 2H), 3.22-2.98 (m, 6H), 2.92 (s, 6H), 2.75-2.66 (m, 2H), 2.59 (t, J = 7.6 Hz, 2H), 2.54-2.47 (m, 2H), 2.24 (t, J = 7.4 Hz, 2H), 2.13 (t, J = 7.2 Hz, 2H), 1.82-1.70 (m, 5H), 1.53-1.41 (m, 5H); HRMS: (m/z) [M + H]+ calcd. for C58H75ClN13O18S2, 1340.4477; found 1340.4466.

Figure VIII.9: Analytical HPLC trace of 7b using a Synergi 4 µm, Polar-RP 150 x 10 mm column at a flow rate of 4 mL/min with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 260 nm.
AAZ Targeted CBI Carbamate – 8a

AAZ targeted charged linker 11a (7.6 mg, 8.3 µmol) and activated carbamate 13 (2.7 mg, 3.8 µmol) were dissolved in degassed MeOH (0.5 mL) and stirred for 6 h at room temperature. The reaction mixture was directly purified over reverse-phased HPLC (95% A / 5% B to 20% A / 80% B over 20 min), fractions containing the product by MS were pooled and lyophilized to give the title compound as a white powder (2.0 mg, 1.3 µmol, 35%).

$^1$H-NMR (500 MHz, DMSO-d$_6$, mixture of 2 rotamers) $\delta$ [ppm] = 13.00 (br s, 1H), 12.39 (br s, 1H), 11.54 (s, 1H), 9.72 (br s, 1H), 8.32 (s, 2H), 8.24-8.18 (m, 3H), 8.14-8.10 (m, 1H), 8.03-7.97 (m, 2H), 7.92-7.86 (m, 1H), 7.83 (s, 1H), 7.62-7.58 (m, 1H), 7.51-7.44 (m, 2H), 7.32-7.01 (m, 7H), 4.85 (t, $J = 10.6$ Hz, 1H), 4.61-4.47 (m, 4H), 4.43-4.38 (m, 1H), 4.30-4.19 (m, 5H), 4.11-4.08 (m, 1H), 3.99-3.89 (m, 2H), 3.85 (s, 3H), 3.63-3.53 (m, 4H), 3.25-2.96 (m, 8H), 2.93 (s, 6H), 2.75-2.57 (m, 6H), 2.53-2.47 (m, 2H), 2.13 (t, $J = 7.0$ Hz, 2H), 1.96-1.90 (m, 2H), 1.78-1.68 (m, 3H), 1.54-1.40 (m, 5H); HRMS: (m/z) [M + 2H]$^{2+}$ calcd. for C$_{61}$H$_{81}$ClN$_{18}$O$_{18}$S$_4$, 758.2268; found 758.2267.

Figure VIII.10: Analytical HPLC trace of 8a using a Synergi 4µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL/min with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 260 nm.
Activated carbamate 13 (5.0 mg, 6.9 µmol) and untargeted charged linker 11b (10 mg, 14 µmol) were dissolved in degassed MeOH (0.5 mL) and stirred for 6 h at room temperature. The reaction mixture was directly purified over reverse-phased HPLC (95% A / 5% B to 20% A / 80% B over 20 min), fractions containing the product by MS were pooled and lyophilized to give the title compound as a white powder (5.1 mg, 3.7 µmol, 54%).

$^1$H-NMR (500 MHz, DMSO-d$_6$, mixture of 2 rotamers) $\delta$ [ppm] = 12.40 (br s, 3H) 11.54 (s, 1H), 8.24-8.14 (m, 4H), 8.03-7.87 (m, 3H), 7.82 (s, 1H), 7.63-7.47 (m, 3H), 7.32-6.82 (m, 7H), 4.85 (t, $J = 10.0$ Hz, 1H), 4.62-4.53 (m, 3H), 4.50-4.44 (br m, 1H), 4.43-4.37 (br m, 1H), 4.30-4.20 (m, 5H), 3.99-3.87 (m, 5H), 3.85 (s, 3H), 3.64-3.50 (m, 4H), 3.25-2.95 (m, 8H), 2.93 (s, 6H), 2.85-2.58 (m, 4H), 2.53-2.46 (m, 2H), 2.25 (t, $J = 7.4$ Hz, 2H), 2.12 (t, $J = 7.3$ Hz, 2H), 1.84-1.68 (m, 5H), 1.56-1.38 (m, 5H); HRMS: (m/z) [M + 2H]$^{2+}$ calcd. for C$_{59}$H$_{79}$ClN$_{14}$O$_{17}$S$_2$, 677.2433; found 677.2430.

Figure VIII.11: Analytical HPLC trace of 8b using a Synergi 4µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL/min with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 260 nm.
CysAspArgAsp-Linker-AAZ 11a (40 mg, 40 µmol) was dissolved in degassed MeOH (5 mL) and 2,2'-dipyridyldisulfide (13.2 mg, 60 µmol) was added. The mixture was stirred at room temperature for 12 h and added drop wise to ice cold diethyl ether (40 mL). The precipitate was collected by centrifugation, re-dissolved in MeOH and precipitated again with ice cold diethyl ether (40 mL) and dried under vacuum to give the activated disulfide as a white residue (20 mg, 20 µmol, 49%). An aliquot of the activated disulfide (8 mg, 7.8 µmol) was dissolved in DMF (500 µL) and DM1 free thiol (5.5 mg, 7.4 µmol) added. The reaction was allowed to stand at room temperature for 48 h after which the product was recovered by reversed phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). Fractions containing the desired product by MS were pooled and lyophilized to yield the title compound as an off white powder (9.0 mg, 5.5 µmol, 74%).

$^1$H-NMR (500 MHz, DMSO-d$_6$) $\delta$ [ppm] = 8.31-8.20 (m, 4H), 7.95-7.85 (m, 4H), 7.37-6.99 (br s, 5H), 7.13 (s, 1H), 6.90 (s, 1H), 6.61-6.52 (m, 3H), 5.55 (dd, $J$ = 14.5, 9.1 Hz, 1H), 5.37-5.28 (m, 1H), 4.55-4.51 (m, 4H), 4.29-4.22 (m, 4H), 4.05 (t, $J$ = 11.8 Hz), 3.91 (s, 1H), 3.49-3.00 (m, integration impossible), 2.90-2.44 (m, integration impossible), 2.13 (t, $J$ = 7.2 Hz), 2.02 (d, $J$ = 12.5 Hz), 1.77-1.73 (m, 5H), 1.57 (s, 3H), 1.55-1.31 (m, 11H), 1.25-1.10 (m, 11H), 0.77 (s, 4H); HRMS: (m/z) [M + 2H]$^{2+}$ calcd. for C$_{65}$H$_{94}$ClN$_{17}$O$_{23}$S$_4$ 821.7634; found 821.7633.
**Figure VIII.12:** Analytical HPLC trace of 9a using a Synergi 4µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL/min with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 260 nm.

**Untargeted DM1 Conjugate – 9b**

CysAspArgAsp-Linker-COOH 11b (21 mg, 28 µmol) was dissolved in degassed MeOH (5 mL) and reduced with TCEP-HCl (16 mg, 56 µmol) for 2 h at room temperature. 2,2'-Dipyridyldisulfide (25 mg, 114 µmol) was added and the mixture stirred for 12 h at room temperature. The reaction was precipitated into ice cold diethyl ether (40 mL), the product collected by centrifugation, re-dissolved in MeOH (5 mL) and precipitated again with ice cold diethyl ether (40 mL). The precipitate was dried under vacuum to give the activated disulfide as a white residue (20 mg, 23 µmol, 83%). An aliquot of the activated disulfide (10 mg, 12 µmol) was dissolved in DMF (500 µL) and DM1 free thiol (8.6 mg, 12 µmol) was added. The reaction was allowed to stand at room temperature for 48 h after which the product was recovered by reversed phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). Fractions containing the desired product by MS were pooled and lyophilized to yield the title compound as an off white powder (7.0 mg, 4.7 µmol, 40%).

$^1$H-NMR (500 MHz, DMSO-d$_6$) δ [ppm] = 8.22 (d, $J = 7.3$ Hz, 2H), 7.97-7.82 (m, 4H), 7.40-6.95 (br s, 4H), 7.13 (s, 1H), 6.91 (s, 1H), 6.58-6.52 (m, 3H), 5.93 (s, 1H), 5.55 (m, 1H), 5.30 (m, 1H), 4.53 (m, 3H), 4.29-4.21 (m, 4H), 4.05 (t, $J = 11.5$ Hz, 1H, 3.90
(s, 3H), 3.59-3.00 (m, integration impossible), 2.91-2.45 (m, integration impossible), 2.25 (t, J = 7.3 Hz, 2H), 2.15-2.11 (m, 2H), 2.03 (d, J = 13.0 Hz, 1H), 1.83-1.69 (m, 5H), 1.57 (s, 2H), 1.55-1.38 (m, 4H), 1.26-1.10 (m, 6H), 0.77 (s, 2H); HRMS: (m/z) [M + 2H]^2+ calcd. for C_{63}H_{92}ClN_{13}O_{22}S_{2}, 740.7799; found 740.7792.

Figure VIII.13: Analytical HPLC trace of 9b using a Synergi 4µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL/min with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 260 nm.

N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)hex-5-ynamide – 10

A solution of 5-hexynoic acid (1.4 mL, 12.9 mmol) and DMF (50 µL) in DCM (50 mL) was cooled on ice and oxalyl chloride (1 mL, 11.7 mmol) was added drop wise over 15 min. The reaction was allowed to warm to room temperature, stirred until evanescence ceased and then concentrated under reduced pressure. The yellow liquid was added drop wise to a solution of 23 (2.3 g, 12.9 mmol) and pyridine (943 µL, 25.8 mmol) in DMF (15 mL) and the reaction stirred for 3 h at room temperature. The solvent was removed under reduced pressure and the residue purified by flash column chromatography (EtOAc) to give the product as an off-white solid (2.8 g, 79%).

$^1$H-NMR (400 MHz, DMSO-d$_6$) δ [ppm] = 13.01 (s, 1H), 8.30 (s, 2H), 2.81 (t, J = 2.6 Hz, 1H), 2.65 (t, J = 7.4 Hz, 2H), 2.24 (td, J = 7.1, 2.6 Hz, 2H), 1.84-1.77 (m, 2H); $^{13}$C-NMR (100 MHz, DMSO-d$_6$) δ [ppm] = 171.5, 164.2, 160.9, 83.6, 71.8, 33.6, 23.1, 17.2; HRMS: (m/z) [M + H]$^+$ calcd. for C$_8$H$_{11}$N$_4$O$_3$S$_6$, 275.0267; found 275.0268.
CysAspArgAsp-Linker-Acetazolamide – 11a

Commercially available pre-loaded Fmoc-Cys(Trt) on Tentagel resin (500 mg, 0.415 mmol, RAPP Polymere) was swollen in DMF (3 × 5 min × 5 mL), the Fmoc group removed with 20 % piperidine in DMF (1 × 1 min × 5 mL and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL). The peptide was extended with Fmoc-Asp(tBu)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Asp(tBu)-OH in the indicated order and then capped with 5-azido-valerate. For this purpose, the Fmoc protected amino acid or azido acid (3.0 eq), HBTU (3.0 eq), HOBt (3.0 eq) and DIPEA (6.0 eq) were dissolved in DMF (5 mL), the mixture was allowed to stand for 1 min at room temperature and then reacted with the resin for 1 h under gentle agitation. After washing with DMF (6 × 1 min × 5 mL) the Fmoc group was removed with 20 % piperidine in DMF (1 × 1 min × 5 min and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL) before the next coupling step was initiated. After coupling of 5-azido-valerate, a solution of Cul (0.3 eq), TBTA (0.3 eq) and alkyne 10 (6 eq) in a mixture of DMF (2.5 mL) and THF (2.5 mL) was prepared and reacted with the resin for 2 h at room temperature. After washing with DMF (3 × 1 min × 5 mL), 50 mM eq. EDTA solution (3 × 1 min × 5 mL), DMF (3 × 1 min × 5 mL) and DCM (3 × 1 min × 5 mL), the compound was cleaved by agitating the resin with a mixture of TFA (4.5 mL), TIS (250 µL) and H₂O (250 µL) for 2 h at room temperature. The resin was washed with TFA (1 × 5 min × 5 mL) and the combined cleavage and washing solutions added drop-wise to ice cold diethyl ether (100 mL). The precipitate was collected by centrifugation and the product purified by reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). After lyophilization the title compound was collected as a white powder (135 mg, 0.14 mmol, 33%).

¹H-NMR (500 MHz, DMSO-d₆) δ [ppm] = 13.00 (s, 1H), 8.31 (s, 2H), 8.23-8.20 (m, 2H), 7.98 (d, J = 7.5 Hz, 1H), 7.93 (d, J = 7.8 Hz, 1H), 7.82 (br m, 1H), 7.53 (br m, 1H), 7.27-7.06 (br m, 4H), 4.59-4.51 (m, 2H), 4.40-4.36 (m, 1H), 4.27 (t, J = 6.7 Hz, 2H), 4.21-4.20 (m, 1H), 3.06-3.04 (br m, 2H), 2.87-2.47 (m, 9H), 2.38 (t, J = 8.6 Hz, 1H), 2.14 (t, J = 7.0 Hz, 2H), 1.95-1.90 (m, 2H), 1.77-1.69 (m, 3H), 1.54-1.39 (m, 5H);

¹³C-NMR (125 MHz, DMSO-d₆) δ [ppm] = 172.7, 172.5, 172.3, 172.1, 171.7, 171.6,
171.5, 170.9, 164.7, 161.5, 157.2, 146.5, 122.3, 54.9, 52.7, 50.1, 49.9, 49.3, 40.9, 36.3, 36.2, 29.6, 29.4, 25.9, 25.2, 24.8, 24.6, 22.5; HRMS: (m/z) [M + H]+ calcd. for C$_{30}$H$_{47}$N$_{14}$O$_{13}$S$_{3}$, 907.2604; found 907.2594.

**CysAspArgAsp-Linker-COOH – 11b**

Commerciially available pre-loaded Fmoc-Cys(Trt) on Tentagel resin (500 mg, 0.415 mmol, RAPP polymere) was swollen in DMF (3 × 5 min × 5 mL), the Fmoc group removed with 20 % piperidine in DMF (1 × 1 min × 5 mL and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL). The peptide was extended with Fmoc-Asp(OtBu)-OH, Fmoc-Arg(Pbf)-OH and Fmpc-Asp(OtBu)-OH in the indicated order and then capped with 5-azido-valerate. For this purpose, the Fmoc protected amino acid or azido acid (3.0 eq), HBTU (3.0 eq), HOBt (3.0 eq) and DIPEA (6.0 eq) were dissolved in DMF (5 mL), the mixture was allowed to stand for 1 min at room temperature and then reacted with the resin for 1 h under gentle agitation. After washing with DMF (6 × 1 min × 5 mL) the Fmoc group was removed with 20 % piperidine in DMF (1 × 1 min × 5 min and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL) before the next coupling step was initiated. After coupling of 5-azido-valerate, a solution of Cul (0.3 eq), TBTA (0.3 eq) and 5-hexynoic acid (6 eq) in a mixture of DMF (2.5 mL) and THF (2.5 mL) was prepared and reacted with the resin for 2 h at room temperature. After washing with DMF (3 × 1 min × 5 mL), 50 mM eq. EDTA solution (3 × 1 min × 5 mL), DMF (3 × 1 min × 5 mL) and DCM (3 × 1 min × 5 mL), the compound was cleaved by agitating the resin with a mixture of TFA (4.4 mL), phenol (250 µL), water (250 µL) and TIPS (100 µL) for 2 h at room temperature. The resin was washed with TFA (1 × 5 min × 5 mL) and the combined cleavage and washing solutions added drop-wise to ice cold diethyl ether (100 mL). The precipitate was collected by centrifugation and the product purified by reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). After lyophilization the title compound was collected as a white powder (116 mg, 0.16 mmol, 43%).
\( ^1H-NMR \) (400 MHz, MeOH-d\(_4\)) \( \delta \) [ppm] = 7.81 (s, 1H), 4.80 (t, \( J = 5.6 \) Hz, 1H) 4.67-4.60 (m, 2H), 4.42-4.38 (m, 3H), 3.21 (t, \( J = 6.9 \) Hz, 2H), 3.00-2.74 (m, 8H), 2.37 (t, \( J = 7.4 \) Hz, 2H), 2.30 (t, \( J = 7.4 \) Hz, 2H), 2.05-1.91 (m, 5H), 1.80-1.60 (m, 5H); \( ^{13}C-NMR \) (125 MHz, DMSO-d\(_6\)) \( \delta \) [ppm] = 174.6, 173.6, 172.7, 172.2, 172.0, 171.6, 171.4, 170.7, 157.1, 146.7, 122.3, 54.8, 52.7, 49.9, 49.8, 49.3, 40.8, 36.2, 34.7, 33.4, 33.1, 29.6, 29.4, 25.8, 25.1, 24.8, 24.7, 22.5; HRMS: (m/z) [M + H]\(^+\) calcd. for C\(_{28}\)H\(_{45}\)N\(_{10}\)O\(_{12}\)S, 745.2934; found 745.2931.

Seco CBI drug carbonate pyridyl disulfide - 12

Seco CBI drug 14 (10 mg, 20 \( \mu \)mol, 1 eq), activated carbonate 16 (7.2 mg, 20 \( \mu \)mol, 1 eq) and \( N,N \)-dimethylaminopyridine (DMAP, 2.4 mg, 50 \( \mu \)mol, 2.5 eq) were dissolved in DMF (2 mL) and stirred for 5 h at room temperature. The reaction mixture was diluted with MeOH (2 mL) and purified over HPLC (95% A / 5% B to 20% A / 80% B over 20 min). Fractions containing the desired product my MS were pooled and lyophilized to yield the title compound as an off white powder (10.1 mg, 14.3 \( \mu \)mol, 72%).

\( ^1H-NMR \) (400 MHz, MeOD-d\(_4\)) \( \delta \) [ppm] = 8.45 (br s, 1H) 8.34 (s, 1H), 7.94-7.81 (m, 4H), 7.56 (dt, \( J = 6.9 \), 1.1 Hz, 1H), 7.48 (dt, \( J = 6.9 \), 1.0 Hz, 1H), 7.33 (s, 1H), 7.26 (m, 1H), 7.06 (s, 1H), 6.99 (s, 1H), 4.70-4.61 (m, 2H), 4.58 (t, \( J = 6.0 \) Hz, 2H), 4.33 (t, \( J = 4.9 \) Hz, 2H), 4.24-4.20 (m, 1H), 4.00 (dd, \( J = 11.4 \), 3.3 Hz, 1H), 3.89 (s, 3H), 3.71 (dd, \( J = 11.3 \), 8.2 Hz, 1H), 3.60 (m, 2H), 3.25 (t, \( J = 6.0 \) Hz, 2H), 3.06 (s, 6H); \( ^{13}C-NMR \) (125 MHz, MeOD-d\(_4\)) \( \delta \) [ppm] = 160.3, 159.3, 153.8, 150.0, 149.2, 146.9, 143.4, 141.2, 137.9, 132.9, 129.7, 128.7, 127.5, 125.1, 123.8, 122.9, 122.7, 121.6, 121.4, 120.6, 120.0, 110.6, 107.8, 106.7, 93.8, 66.3, 64.5, 56.6, 54.8, 54.7, 46.3, 42.5, 42.0, 37.0; HRMS: (m/z) [M + H]\(^+\) calcd. for C\(_{36}\)H\(_{56}\)ClN\(_4\)O\(_6\)S\(_2\), 707.1759; found 707.1761.
Seco CBI Drug carbamate pyridyl disulfide - 13

Seco CBI drug 14 (10 mg, 20 µmol, 1.0 eq), activated carbamate 17 (12 mg, 92 µmol, 4.6 eq) and DMAP (10 mg, 100 µmol, 5.0 eq) were dissolved in DMF (2 mL) and stirred for 12 h at room temperature. The reaction mixture was diluted with MeOH (2 mL) and purified over HPLC (95% A / 5% B to 20% A / 80% B over 20 min). Fractions containing the desired product by MS were pooled and lyophilized to yield the title compound as an off white powder (9.5 mg, 13 µmol, 65%).

1H-NMR (400 MHz, MeOD-d$_4$, mixture of two rotamers) δ [ppm] = 8.42-8.23 (br m, 2H), 7.96-7.73 (m, 4H), 7.32-7.14 (m, 1H), 7.04 (2s, 1H), 6.91 & 6.85 (2s, 1H), 4.67-4.52 (m, 2H), 4.31 (t, J = 4.7 Hz, 2H), 4.17 (t, J = 4.9 Hz, 2H), 3.84-3.73 (m, 4H), 3.70-3.62 (m, 1H), 3.60 (t, J = 4.9 Hz, 2H), 3.36-3.11 (m, 5H), 3.05 (s, 6H); 13C-NMR (125 MHz, MeOD-d$_4$) δ [ppm] = 161.8, 161.7, 161.1, 160.8, 157.0 156.6, 151.5, 150.6, 149.1, 144.9, 142.9, 142.8, 139.1, 134.6, 131.3, 128.8, 126.3, 124.1, 123.8, 123.7, 123.6, 122.6, 122.2, 121.5, 121.5, 112.9, 112.6, 109.5, 108.0, 107.9, 95.5, 98.4, 66.2, 58.1, 56.4, 56.3, 47.8, 44.0, 43.6, 37.5, 37.4, 35.8, 35.7; HRMS: (m/z) [M + H]$^+$ calcd. for C$_{36}$H$_{39}$ClN$_5$O$_5$S$_2$, 720.2076; found 720.2074.

Seco CBI Drug – 14

N-Boc protected seco CBI (50 mg, 150 µmol, 1.0 eq) was dissolved in 4 M HCl in dry EtOAc (5 mL) and stirred for 6 h at room temperature. The solvent was removed under reduced pressure and the residue dissolved in 3 mL DMF and cooled on ice. EDC-HCl (86 mg, 450 µmol, 3.0 eq) was added followed by indole 18 (61 mg, 220
µmol, 1.3 eq), the mixture warmed to room temperature and allowed to stir for 12 h. MeOH (3 mL) was added and the crude reaction mixture purified over HPLC (95% A / 5% B to 20% A / 80% B over 20 min). Fractions containing the desired product by MS were pooled and lyophilized to yield the title compound as an off-yellow powder (44.1 mg, 89.5 µmol, 60%).

$^1$H-NMR (400 MHz, MeOD-d4) δ [ppm] = 8.22 (d, $J = 8.2$ Hz, 1H), 7.87 (s, 1H), 7.77 (d, $J = 8.3$ Hz, 1H), 7.53 (td, $J = 6.8$, 1.2 Hz, 1H), 7.37 (td, $J = 6.8$, 1.0 Hz, 1H), 7.33 (s, 1H), 7.10 (s, 1H), 7.07 (s, 1H), 4.67-4.64 (m, 2H), 4.31 (t, $J = 4.8$ Hz, 2H), 4.15-4.11 (m, 1H), 3.98 (dd, $J = 11.2$, 3.2 Hz, 1H), 3.92 (s, 3H), 3.62-3.56 (m, 3H), 3.06 (s, 6H); $^{13}$C-NMR (100 MHz, MeOD-d4) δ [ppm] = 173.0, 162.4, 155.8, 151.6, 144.9, 143.4, 134.6, 131.6, 130.9, 128.6, 124.6, 124.5, 123.5, 122.2, 117.0, 109.5, 107.9, 101.5, 95.5, 66.0, 58.1, 56.8, 56.3, 47.5, 43.9, 43.6; HRMS: (m/z) [M + H]$^+$ calcd. for C$_{27}$H$_{29}$ClN$_3$O$_4$, 494.1841; found 494.1843.

$N1$-(2-(2-(aminoethoxy)ethoxy)ethyl)-$N4$-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)succinamide – 25

![Reaction Scheme](image)

Commercially available pre-loaded O-Bis-(aminoethyl)ethylene glycol on trityl resin (500 mg, 0.3 mmol, Merck Millipore) was swollen in DMF (3 × 5 min × 5 mL). 4-oxo-4-((5-sulfamoyl-1,3,4-thiadiazol-2-yl)amino)butanoic acid (AAZSucc, 166 mg, 0.59 mmol) and HATU (228 mg, 0.60 mmol) were dissolved in DMF (5 mL) and DIPEA (200 µL, 1.2 mmol) was added. The solution was immediately reacted with the resin for 30 min at room temperature. The resin was washed with DMF (6 × 1 min × 5 mL), DCM (3 × 1 min × 5 mL) and cleaved with 95% TFA / 2.5% H$_2$O / 2.5% triisopropylsilane (TIS, 5 mL total volume) for 1 h at room temperature and washed with TFA (1 × 1 min × 5 mL). The combined cleavage and wash solutions were poured into cold Et$_2$O (40 mL), the precipitate collected by centrifugation and purified over reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). Fractions containing the desired product by MS were pooled and lyophilized to give the product as a white powder (48 mg, 0.12 mmol, 39%).
**1H-NMR (400 MHz, MeOD-d$_4$) δ [ppm] = 3.72 (t, $J = 4.3$ Hz, 2H), 3.69-3.66 (m, 4H), 3.57 (t, $J = 5.7$ Hz, 2H), 3.39 (t, $J = 5.6$ Hz, 2H), 3.14 (t, $J = 5.6$ Hz, 2H), 2.87 (t, $J = 5.6$ Hz, 2H), 2.66 (t, $J = 6.2$ Hz, 2H); $^{13}$C-NMR (100 MHz, MeOD-d$_4$) δ [ppm] = 174.3, 173.0, 166.4, 163.1, 71.4, 71.3, 70.7, 67.9, 40.7, 40.3, 31.5, 30.9; HRMS: (m/z) [M + H]$^+$ calcd. for C$_{12}$H$_{23}$N$_6$O$_6$S$_2$, 411.1115; found 411.1116.

$(S)$-**tert-butyl (2-(2-(3-methyl-2-(4-(4-sulfamoylphenyl)-1H-1,2,3-triazol-1-yl)butanamido)ethoxy)ethoxy)ethyl)carbamate – 26

![Chemical Structure](image)

To a solution of 27 (64 mg, 0.20 mmol) in DMF (2 mL) was added NHS (25 mg, 0.22 mmol) and EDC.HCl (42 mg, 0.22 mmol) and the mixture was stirred for 1 h at room temperature. A solution of 19 (54 mg, 0.22 mmol) and DIPEA (110 µL, 0.67 mmol) in DMF (1 mL) was added and the reaction stirred for 1 h at room temperature. The solvent was removed under reduced pressure, the residue dissolved in DCM (5 mL) and the solution washed with H$_2$O (1 × 5 mL), brine (1 × 5 mL), dried over Na$_2$SO$_4$ and the solvent removed under reduced pressure. Purification by flash column chromatography over silica (EtOAc) gave the product as a white solid (63 mg, 0.11 mmol, 57%).

$^1$H-NMR (400 MHz, MeOD-d$_4$) δ [ppm] = 8.75 (br m, 1H), 8.69 (s, 1H), 8.05 (d, $J = 6.6$ Hz, 2H), 7.98 (d, $J = 6.6$ Hz, 2H), 5.03 (d, $J = 10.4$ Hz, 1H), 3.61-3.56 (m, 6H), 3.53-3.37 (m, 4H), 3.22 (t, $J = 5.6$ Hz, 2H), 2.65-2.56 (m, 1H), 1.44 (s, 9H), 1.12 (d, $J = 6.7$ Hz, 3H), 0.84 (d, $J = 6.6$ Hz, 3H); $^{13}$C-NMR (100 MHz, MeOD-d$_4$) δ [ppm] = 170.0, 158.5, 147.5, 144.5, 135.5, 128.0, 127.0, 122.3, 80.1, 71.7, 71.6, 71.3, 71.1, 70.3, 41.2, 40.7, 33.0, 28.8, 19.6, 19.2; HRMS: (m/z) [M + H]$^+$ calcd. for C$_{24}$H$_{38}$N$_6$NaO$_7$S, 577.2415; meas. 577.2415.

$(S)$-3-methyl-2-(4-(4-sulfamoylphenyl)-1H-1,2,3-triazol-1-yl)butanoic acid - 27

![Chemical Structure](image)
Ethynyl benzene sulfonylamine (54 mg, 0.3 mmol), azido valine 20 (42 mg, 0.3 mmol) and tris-(benzyltriazolylmethyl)amine (TBTA, 0.3 mg, cat.) were dissolved in a mixture of tBuOH (3.7 mL), a 0.04 M CuSO₄ solution in PBS pH 7.4 (2.0 mL) and a 0.1 M sodium ascorbate solution in PBS pH 7.4 (1.7 mL) and stirred for 12 h at room temperature. All solvents had previously been de-gassed and flushed with Ar. The reaction was poured onto 25 mL H₂O acidified to pH 2.0 and the mixture extracted with EtOAc (4 × 20 mL), the pooled organic phases washed with brine and dried over MgSO₄. The solvent was removed under vacuum and the residue purified over silica (20% MeOH in DCM with 0.1% Et₃N) to yield the product as a white solid (70 mg, 72%).

**1H-NMR** (400 MHz, DMSO-d₆) δ [ppm] = 8.85 (s, 1H), 8.10 (d, J = 8.5 Hz, 2H), 7.90 (d, J = 8.5 Hz, 2H), 7.39 (s, 2), 5.24 (d, J = 8.0 Hz, 1H), 2.64-2.55 (m, 1H), 1.00 (d, J = 6.7 Hz, 3H), 0.88 (d, J = 6.7 Hz, 3H); **13C-NMR** (100 MHz, DMSO-d₆) δ [ppm] = 169.6, 145.0, 143.2, 133.7, 126.3, 125.4, 122.6, 68.3, 30.4, 19.0, 18.3; HRMS: (m/z) [M + H]⁺ calcd. for C₁₃H₁₅N₄Na₂O₄S, 369.0604; found 369.0609.

**tert-butyl (2-(2-((4-chloro-6-((4-sulfamoylphenyl)amino)-1,3,5-triazin-2-yl)amino)ethoxy)ethoxy)ethyl)carbamate – 28**

21 (160 mg, 0.64 mmol), 14 (204 mg, 0.64 mmol) and DIPEA (105 µL, 0.64 mmol) were dissolved in DMF (5 mL) and stirred for 3 h at room temperature. The reaction mixture was diluted with H₂O (15 mL) and extracted with EtOAc (3 × 10 mL). The combined organic fractions were washed with 10% w/v aq. LiCl solution (1 × 10 mL), brine (1 × 10 mL) and dried over Na₂SO₄. The solvent was removed under reduced pressure and the residue purified over silica (EtOAc) to give the product as a white solid (239 mg, 66%).

**1H-NMR** (400 MHz, DMSO-d₆, mixture of two rotamers) δ [ppm] = 10.39-10.30 (m, 1H), 8.27 (br s, 1H), 7.93 (d, J = 8.1 Hz, 1H), 7.85 (d, J = 8.1 Hz, 1H), 7.74 (m, 2H), 7.25 (s, 1H), 7.23 (s, 1H), 6.74-6.73 (m, 1H), 3.57-3.32 (m, 10H), 3.05 (m, 2H), 1.37 (s, 9H); **13C-NMR** (125 MHz, DMSO-d₆, mixture of two rotamers, several signals overlap) δ [ppm] = 169.0, 168.5, 166.0, 165.9, 164.2, 163.7, 156.0, 142.6, 142.5,
138.3, 138.2, 127.1, 126.8, 120.0, 119.8, 78.1, 70.1, 70.0, 69.9, 69.6, 69.1, 68.8, 67.1, 39.0, 28.7; HRMS: (m/z) [M + H]+ calcd. for C_{20}H_{30}ClN_{7}NaO_{6}S, 554.1559; found 554.1555.

(E)-tert-butyl (2-(2-(3-((4-(4-sulfamoylphenyl)diazenyl)phenyl)amino)propanamido)ethoxy)ethoxy)ethyl)carbamate – 29

To 30 (20 mg, 57 µmol) dissolved in DMF (1 mL) was added HOBt (8.7 mg, 57 µmol) followed by EDC·HCl (12.2 mg, 64 µmol). After stirring the reaction for 1 h at room temperature a solution of 19 (15.8 mg, 64 mmol) and DIPEA (20 µL, 122 µmol) in DMF (0.5 mL) was added. The mixture was stirred for 1 h at room temperature, diluted with MeOH (1.5 mL) and purified over reversed-phase HPLC (80% A / 20% B to 20% A / 80% B over 20 min). Fractions containing the desired product by MS were pooled and lyophilized to give the product as an orange powder (26 mg, 45 mmol, 79%).

^1^H-NMR (400 MHz, MeOD-d4) δ [ppm] = 8.02 (d, J = 8.8 Hz, 2H), 7.91 (d, J = 8.8 Hz, 2H), 7.83 (d, J = 9.0 Hz, 2H), 6.76 (d, J = 9.0 Hz, 2H), 3.60 (s, 4H), 3.58-3.49 (m, 6H), 3.40 (t, J = 5.5 Hz, 2H), 3.23-3.20 (m, 2H), 2.56 (t, J = 6.8 Hz, 2H), 1.45 (s, 9H); ^13^C-NMR (125 MHz, DMSO-d6) δ [ppm] = 170.8, 156.1, 154.7, 153.2, 144.3, 143.3, 127.4, 126.3, 122.4, 112.2, 78.1, 70.0, 69.9, 69.6, 69.5, 39.5, 39.4, 39.1, 35.4, 28.7; HRMS: (m/z) [M + H]+ calcd. for C_{26}H_{38}N_{6}NaO_{7}S, 601.2415; found 601.2416.

(E)-3-((4-(4-sulfamoylphenyl)diazenyl)phenyl)amino)propanoic acid – 30

Sulfanilamide (85 mg, 0.49 mmol) was dissolved in 40% aq. HCl (1.3 mL) and cooled to 0 °C. A solution of NaNO₂ in water (300 µL) was added drop-wise over 5 min and the reaction stirred on ice for 15 min. The yellowish solution was slowly added to a suspension of 22 (129 mg, 0.37 mmol) in 10M aq. NaOH (1 mL) and DMF (1 mL) and stirred for 2 h at room temperature. The dark red solution was acidified with 6 N HCl,
extracted with EtOAc (6 × 10 mL), dried over Na$_2$SO$_4$ and the solvent removed under reduced pressure. Recrystallization of the dark red residue gave the product as a red solid (32 mg, 25%).

$^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$ [ppm] = 7.95 (d, $J = 8.6$ Hz, 2H), 7.88 (d, $J = 8.6$ Hz, 2H), 7.77 (d, $J = 8.9$ Hz, 2H), 7.44 (s, 2H), 6.94 (br s, 1H), 6.74 (d, $J = 8.8$ Hz, 2H), 3.40 (t, $J = 6.4$ Hz, 2H), 2.56 (t, $J = 6.4$ Hz, 2H); $^{13}$C-NMR (100 MHz, DMSO-d$_6$) $\delta$ [ppm] = 172.9, 154.1, 152.6, 143.8, 142.9, 126.8, 125.8, 121.9, 111.7, 38.4, 33.4; HRMS: (m/z) [M + H]$^+$ calcd. for C$_{15}$H$_{17}$N$_4$O$_4$S, 349.0965; found 349.0967.

tert-butyl (2-(2-(4-sulfamoylbenzamido)ethoxy)ethoxy)ethyl)carbamate - 31

To a solution of 4-carboxybenzenesulfonamide (46 mg, 0.23 mmol) in MeCN (2 mL) was added NHS (29 mg, 0.25 mmol) followed by EDC-HCl (48 mg, 0.25 mmol). After stirring for 4 h at room temperature more EDC-HCl (24 mg, 0.13 mmol) was added and the reaction stirred for a further 1 h at room temperature. A solution of 19 (52 mg, 0.21 mmol) and DIPEA (140 µL, 0.85 mmol) in DMF (1 mL) was added. After stirring for 10 h at room temperature, the reaction was filtered through a pad of silica eluting with EtOAc, the solvent removed under reduced pressure and the residue purified over silica (EtOAc to 10% MeOH in EtOAc) to give the product as a white solid (61 mg, 0.14 mmol, 67%).

$^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$ [ppm] = 8.71 (t, $J = 5.6$ Hz, 1H), 8.00 (d, $J = 8.0$ Hz, 2H), 7.90 (d, $J = 8.0$ Hz, 2H), 7.46 (br s, 2H), 6.76 (t, $J = 5.2$ Hz, 1H), 3.56-3.37 (m, 10H), 3.06-3.05 (m, 2H), 1.37 (s, 9H); $^{13}$C-NMR (125 MHz, DMSO-d$_6$) $\delta$ [ppm] = 165.8, 156.1, 146.7, 137.7, 128.3, 126.1, 78.1, 70.0, 69.9, 69.6, 69.2, 67.1, 39.1, 28.7; HRMS: (m/z) [M + H]$^+$ calcd. for C$_{18}$H$_{29}$N$_3$NaO$_3$S, 454.1618; found 454.1623.
2.4. NMR Spectra

400 MHz proton spectrum of 1a in MeOD-\(d_4\)

125 MHz carbon spectrum of 1a in DMSO-\(d_6\)
500 MHz proton spectrum of 2a in DMSO-d6

125 MHz carbon spectrum of 2a in DMSO-d6
400 MHz proton spectrum of 3a in DMSO-d6

125 MHz carbon spectrum of 3a in DMSO-d6
500 MHz proton spectrum of 5a in DMSO-d6

125 MHz carbon spectrum of 5a in DMSO-d6
500 MHz proton spectrum of 7a in DMSO-d6

500 MHz proton spectrum of 7b in DMSO-d6
500 MHz proton spectrum of 8a in DMSO-d6

500 MHz proton spectrum of 8b in DMSO-d6
500 MHz proton spectrum of 9a in DMSO-d6

500 MHz proton spectrum of 9b in DMSO-d6
400 MHz proton spectrum of 10 in DMSO-d6

100 MHz carbon spectrum of 10 in DMSO-d6
500 MHz proton spectrum of 11a in DMSO-d6

125 MHz carbon spectrum of 11a in DMSO-d6
400 MHz proton spectrum of 11b in MeOD-d4

125 MHz carbon spectrum of 11b in DMSO-d6
400 MHz proton spectrum of 12 in MeOD-d4

125 MHz proton spectrum of 12 in MeOD-d4
400 MHz proton spectrum of 13 in MeOD-d4

125 MHz carbon spectrum of 13 in MeOD-d4
400 MHz proton spectrum of 14 in MeOD-d4

100 MHz carbon spectrum of 14 in MeOD-d4
400 MHz spectrum of 25 in MeOD-d4

100 MHz carbon spectrum of 25 in MeOD-d
400 MHz proton spectrum of 26 in MeOD-d4

100 MHz carbon spectrum of 26 in MeOD-d6
400 MHz proton spectrum of 27 in DMSO-d6

100 MHz carbon spectrum of 27 in DMSO-d6
400 MHz proton spectrum of 28 in DMSO-d6

125 MHz carbon spectrum of 28 in DMSO-d6
400 MHz proton spectrum of 29 in MeOD-d4

125 MHz carbon spectrum of 29 in DMSO-d6
400 MHz proton spectrum of 30 in DMSO-d6

100 MHz carbon spectrum of 30 in DMSO-d6
400 MHz proton spectrum of 31 in DMSO-d6

125 MHz carbon spectrum of 31 in DMSO-d6
IX. APPENDIX II: BIVALENT SMALL MOLECULE-DRUG CONJUGATES DIRECTED AGAINST CARBONIC ANHYDRASE IX – EXPERIMENTAL PROCEDURES

This section corresponds to the experimental procedures of the journal article entitled “A bivalent small molecule-drug conjugate directed against carbonic anhydrase IX can elicit complete tumour regression in mice” published in Chemical Science in 2014[60].

1. Biological Procedures

1.1. Propagation of Errors

Standard errors and standard deviations were propagated during data analysis using the method recommended by the National Institute of Standards and Technology.[361] In general, the standard deviation $\sigma_f$ of $f = f(x_1, x_2, \ldots, x_n)$ is given by formula (1) where $\sigma_i$ is the standard deviation of $x_i$.

$$
\sigma_f = \sqrt{\sum_{i=1..n} \left( \frac{\partial f}{\partial x_i} \sigma_i \right)^2} \quad (1)
$$

1.2. Affinity Determination of CAIX Ligands by Surface Plasmon Resonance

Surface plasmon resonance (SPR) experiments were performed on a Biacore T200 instrument (GE Healthcare) at room temperature. For all measurements, CM5 chips and flow buffer of PBS pH 7.4 with DMSO (5 % v/v) and P20 surfactant (0.05 % v/v, GE Healthcare) were used. CAIX protein was immobilised on the chip to 3,000 – 4,000 response units (RU) using EDC·HCl and NHS following manufacturer instructions. Serial dilutions of monovalent ligand 1 or bivalent ligand 2 (640 to 0.63 nM in steps of 1:4) in running buffer at a flow rate of 25 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 H$_2$O. For bivalent ligand 2 an additional washing step with aqueous HCl (50 mM) for 30 sec at 25 µL min$^{-1}$ was required to remove bound ligand. Sensorgrams were solvent corrected and the binding kinetics were analyzed with the Biacore T200 evaluation software (version 2.0) using a 1:1 Langmuir or bivalent binding model. Matlab (R2010b) was used for generation of sensogram plots from exported data.
1.3. **Cell Culture**

SKRC52 and HEK cells were cultured in RPMI medium as described previously. A375 cells were cultured in RPMI medium supplemented with 10% fetal calf serum (FCS, Invitrogen) and antibiotic-antimycotic (Invitrogen) at 37 °C with 5% CO₂. When reaching 90% confluence, cells were detached using 0.05% EDTA-Trypsin (Invitrogen) and re-seeded at a dilution of 1:4.

1.4. **Ligand Binding Analysis by Flow Cytometry**

Binding of ligand-IRDye750 conjugates 3, 4, 5 or 6 to CAIX-expressing SKRC52 human kidney cancer cells and CAIX-negative HEK cells was analyzed by flow cytometry as described previously. In brief, cells were incubated with dye conjugates in PBS pH 7.4 containing 1% FCS v/v for 1h at 0 °C, washed, resuspended in PBS pH 7.4 containing 1% FCS v/v and propidium iodide as a viability dye and analyzed by flow-cytometry.

1.5. **Immunofluorescence Microscopy**

Tumor specimens were shock frozen in NEG 50 (Thermo Scientific) and stored at -80 °C for up to 2 days before use. Organs were warmed to -20 °C and 10 µm sections prepared in a cryostat. Tumor sections were fixed for 10 min in ice-cold acetone, dried and incubated in blocking buffer consisting of 10% FCS v/v and 3% BSA in PBS pH 7.4 for 45 min at room temperature. Slides were washed twice for 5 min in PBS pH 7.4 and incubated with the PHB11 proprietary fully human anti CAIX antibody in IgG format (Philochem, 5 µg mL⁻¹) or the KSF fully human negative control antibody specific for hen egg-white lysozyme in IgG format (Philochem, 5 µg mL⁻¹) in blocking buffer for 90 min at room temperature. After washing (2× 5min with PBS pH 7.4), slides were incubated with a mixture of rat anti-mouse CD31 IgG (BD Pharmigen, 1:500) and rabbit anti-human IgG (Bethyl Laboratories, 1:250) in blocking buffer for 60 min at room temperature. Slides were washed again (2× 5min with PBS pH 7.4), stained with goat anti rabbit Alexa488 conjugates IgG (Invitrogen, 1:200) and donkey anti rat Alexa594 IgG (Invitrogen, 1:200) in blocking buffer for 60 min at room temperature, washed again (2× 5min with PBS pH 7.4), stained with Hoechst 33342 (Invitrogen, 1:1000) in PBS pH 7.4 for 1 min at room temperature, washed (5
min with PBS pH 7.4) and mounted with fluorescence mounting medium (Dako). After drying at room temperature over night, images were taken on a Zeiss Axioskop 2 (Carl Zeiss).

1.6. **In Vitro Cytotoxicity Assay**

In vitro cytotoxicity assays were carried out as described previously.\cite{ref59} In brief, cells were incubated with medium containing varying concentrations of drug conjugates 7 or 8 for 72 h and cell viability determined with MTS reagent (Promega). Alternatively, cells were incubated with drug conjugates for 1 h, washed and then incubated with fresh medium for 71 h followed by viability assessment. Viability was plotted as fraction of cells alive relative to conjugate concentration and the dose of the half-maximum effect ($EC_{50}$) determined by non-linear curve fitting to the four-parameter logistic curve.

1.7. **Stability Determination by High-Performance Liquid Chromatography**

Stability of drug conjugate 7 was determined by High-Performance Liquid Chromatography (HPLC) using a previously described method.\cite{ref59} In brief, drug conjugate 7 was incubated in PBS pH 7.4 or mouse serum (Invitrogen) at 37 °C. Aliquots were withdrawn at different time points, serum proteins precipitated with MeCN and samples analyzed by HPLC. The fraction of intact conjugate was determined by integration over the corresponding intact conjugate peak and divided by the integral at time zero. Etodolac was used as an internal standard.

1.8. **Animal Studies**

All animal experiments were conducted in accordance with Swiss animal welfare laws and regulations under the license number 42/2012 granted by Veterinaeramt des Kanton Zurich.

1.9. **Implantation of Subcutaneous Tumours**

$10^7$ SKRC52 or A375 cells per animal were implanted subcutaneously into the lower backs of balb/c nu/nu mice (Charles River) according to previously established procedures.\cite{ref59}
1.10. Near Infrared Imaging of Tumour Bearing Mice
A modified version of a previously described protocol was used. As reported, mice bearing subcutaneous SKRC52 or A375 tumours (200 – 300 \text{mm}^3 \text{ in size}) were injected with IRDye750 (Licor) labeled CAIX ligands 3-6 (3 nmol) dissolved in 5\% v/v DMSO in PBS pH 7.4 (150 \muL) through the tail vein. Fluorescence images were acquired at 2 h, 4 h, 8 h, 12 h and 24 h after injection on an IVIS Spectrum imaging system (Xenogen, exposure 1s, binning 'small', excitation at 745 nm, emission filter at 800 nm, f number 2, field of view 'C', sample height 1.50 cm). The temperature in the imaging chamber was set to 37 °C and mice were under isoflurane anesthesia during all imaging procedures. Between time points, mice were kept in standard IVC rack cages at 21-24 °C. Food and water were freely available during the course of the entire experiment. After the last time point, mice were sacrificed using CO₂.
Organs were extracted (heart, lung, kidney, liver, spleen, a section of the small intestine of 100 - 150 mg, skeletal muscle weighing in total 100 – 150 mg, stomach, 100 \muL of blood and tumour). Organs were weighed and imaged individually using the IVIS parameters described above. Total fluorescence originating from tumour, muscle or extracted organs was measured by integrating over organ areas on near infrared images using the built in ROI tools of Living Image software version 4.3.1 (Caliper Life Science) and corrected for background fluorescence by subtracting a background ROI of equal size and shape.

1.11. Biodistribution Analysis
A previously described protocol was used with minor modifications. Organs from imaging experiments (24 h time point) were cut into small pieces and ice-cold homogenisation buffer containing EDTA (40 mM), trypsin (6 mg/ml), Triton X-100 (1.6 \muL/ml) and trace amounts of DNase 1 in PBS pH 7.4 (100 \muL per 100 mg of tissue) was added. After treatment on a TissueLyser organ homogeniser (Quiagen, 25 Hz, 15 min), homogenates were incubated for 2 h at room temperature and 100 \muL of each sample was transferred to black 96-well plates. Organ-specific calibration series were prepared by spiking organ homogenates from previously untreated mice with different amounts of IRDye750 (75 pmol – 4.7 pmol dye in 100 \muL of organ homogenate in steps of 1:2) and spotted alongside organ homogenates with unknown dye content. Fluorescent images of plates were recorded on an IVIS
Spectrum imaging system (Xenogen, parameters as above except for height which was set to 0.50 cm) and the fluorescence originating from individual wells was quantified using the built-in plate region of interest (ROI) tool of Living Image software version 4.3.1 (Caliper Life Science). Organ-specific calibration curves were obtained by plotting fluorescence intensity from calibration wells against dye concentration and linear regression analysis. Dye concentrations in organ samples were inferred by comparison of fluorescence intensities from the corresponding well with the calibration curve from the correct organ and then converted to % of injected dose per gram of tissue (% ID g⁻¹, 100 % ID = 3 nmol).

1.12. Therapy Experiments
SKRC52 xenograft bearing balb/c nu/nu mice (Charles River) were randomly assigned into therapy groups of 5 or 6 animals 10 days after tumour implantation. Starting with the day of randomisation, animals were treated with once-daily intravenous injections of 35 nmol 2, 7 or 8 in PBS pH 7.4 (150 µL) containing 5% DMSO for 8 consecutive days. One group was treated with vehicle (5% DMSO in PBS pH 7.4). In the case of the untargeted conjugate an equimolar amount of AAZ was added to the injection solution to control for a possible antitumour activity of CAIX inhibitors. Since sorafenib and sunitinib had previously been shown to lack any measurable anti-tumour effect in this model, these drugs were not included as further controls. Animal weight and tumour dimensions were monitored on a daily basis. Tumour volume was calculated according to the formula \( \text{tumour volume} = \text{long side of tumour} \times \text{short side of tumour}^2 \times 0.5 \). Animals were euthanised when the body weight fell by > 15% relative to the first therapy day or when tumours exceeded a volume of 2000 mm³. Prism 6 (GraphPad Software) was used for statistical data analysis (regular two-way ANOVA with Bonferroni multiple comparison test).

2. Chemical Procedures

2.1. General Chemical Procedures
Proton \( (^1\text{H}) \) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV400 (400 MHz) or a Bruker AVIII500 (500 MHz) spectrometer. Carbon \( (^{13}\text{C}) \) NMR spectra were recorded on a Bruker AV400 (100 MHz) spectrometer or on a Bruker
AVIII500 (125 MHz) spectrometer. Chemical 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 were recorded at ETH’s mass spectrometry service using a Bruker Daltronics maXis ESI-QTOF mass spectrometer. Chemdraw (Perkin Elmer) was used to calculate exact masses. Calculated and exact m/z values are reported in Daltons.

Analytical and preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) were performed on an Waters Alliance HT RP-HPLC with a PDA UV detector, using a Synergi 4µm, Polar-RP 150 × 10 mm 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) at a column temperature of 30 °C.

Anhydrous solvents for reactions were purchased from Acros or Fluka. Peptide grade dimethyl formamide (DMF) for solid phase synthesis was bought from ABCR. All other solvents were used as supplied by Fisher Chemicals, Merck or Aldrich in HPLC or analytical grade. IRDye750 N-hydroxysuccinimidyl (NHS) ester was purchased from Licor, DM1 was purchased from Concortis Biosystems. All other reagents were purchased from 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.

2.2. Synthesis of New Compounds

*Synthesis of AAZTL - 1*

![Chemical structure](image)

Commercially available polystyrene Wang p-nitrophenyl carbonate resin (250 mg, 0.15 mmol) was swollen in DMF (5 mL for 5 min) and reacted with a solution of 2,2’-(ethane-1,2-diylbis(oxy))diethanamine (250 µL), DIPEA (500 µL) and DMAP (2.5 mg) in DMF (4.5 mL) for 12 h at room temperature under shaking. The resin was washed with DMF (3× 5 mL for 1 min), MeOH (3× 5 mL for 1 min) and again DMF (3× 5 mL for 1 min). A solution of 5-azido valeric acid (65 mg, 0.45 mmol), HATU (171 mg,
0.45 mmol) and DIPEA (148 µL, 0.9 mmol) was prepared and immediately reacted with the resin for 1 h at room temperature under shaking. After washing with DMF (6 × 1 min × 5 mL) a solution of Cul (2.9 mg, 0.015 mmol), TBTA (8 mg, 0.015 mmol) and alkyne 13 (123 mg, 0.45 mmol) in a mixture of DMF (1 mL) and THF (1 mL) was prepared and reacted with the resin for 24 h at room temperature. After washing with DMF (3 × 1 min × 5 mL), 50 mM aq. EDTA solution (3 × 1 min × 5 mL), DMF (3 × 1 min × 5 mL) and DCM (3 × 1 min × 5 mL), the compound was cleaved by agitating the resin with a mixture of TFA (2.2 mL), TIS (50 µL), H2O (50 µL), m-cresol (100 µL) and thioanisol (100 µL) for 2 h at room temperature. The resin was washed with TFA (1 × 5 min × 2.5 mL) and the combined cleavage and washing solutions added drop-wise to ice cold diethyl ether (100 mL). The precipitate was collected by centrifugation and the product purified by reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). After lyophilisation the title compound was collected as a white powder (78 mg, 0.14 mmol, 95%).

\[
\text{H-NMR (500 MHz, DMSO-d}_6\text{)} \delta \text{ [ppm] } = \text{13.01 (s, 1H), 8.32 (s, 2H), 7.89-7.82 (m, 5H), 4.28 (t, } J = 7.0 \text{ Hz, 2H), 3.58-3.50 (m, 6H), 3.38 (t, } J = 6.1 \text{ Hz, 2H), 3.18 (m, 2H), 3.00 (m, } 2\text{H), 2.65 (t, } J = 7.5 \text{ Hz, 2H), 2.59 (t, } J = 7.4 \text{ Hz, 2H), 2.09 (t, } J = 7.4 \text{ Hz, 2H), 1.94 (m, } 2\text{H), 1.75 (m, } 2\text{H), 1.42 (m, } 2\text{H); C-NMR (125 MHz, DMSO-d}_6\text{)} \delta \text{ [ppm] } = 172.5, 172.4, 164.8, 161.5, 146.4, 122.4, 70.1, 69.8, 69.6, 67.1, 49.4, 39.1, 38.8,35.0, 34.7, 29.8, 24.8, 24.6, 22.6; \text{HRMS: (m/z) [M + H]}^+ \text{calcd. for C}_{19}\text{H}_{34}\text{N}_{9}\text{O}_{6}\text{S}_{2} 548.2068; \text{found 548.2071.}
\]

**Bivalent targeted amine - 2**

Commercially available polystyrene Wang p-nitrophenyl carbonate resin (500 mg, 0.3 mmol) was swollen in DMF (5 mL for 5 min) and reacted with a solution of 2,2'- (ethane-1,2-diylbis(oxy))diethanamine (500 µL), DIPEA (500 µL) and DMAP (5 mg) in DMF (4 mL) for 12 h at room temperature under shaking. The resin was washed with
DMF (3× 5 mL for 1 min), MeOH (3× 5 mL for 1 min) and again DMF (3× 5 mL for 1 min). A solution of Fmoc-Lys(Fmoc)-OH (532 mg, 0.9 mmol), HBTU (341 mg, 0.9 mmol), HOBt (138 mg, 0.9 mmol) and DIPEA (298 µL, 1.8 mmol) was prepared and immediately reacted with the resin for 1 h at room temperature under shaking. After washing with DMF (6 × 1 min × 5 mL) the Fmoc group was removed with 20 % piperidine in DMF (1 × 1 min × 5 min and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL) before the next coupling step was initiated. In the following, the peptide was extended with Fmoc-Asp(OtBu)-OH twice followed by 5-azido-valerate. For this purpose, a solution of acid (1.2 mmol), HATU (465 mg, 1.2 mmol) and DIPEA (397 µL, 2.4 mmol) was prepared in DMF (5 mL) and reacted with the resin for 1 h at room temperature under gentle agitation. Each coupling was followed by a washing step with DMF (6 × 1 min × 5 mL) and Fmoc deprotection as described above. After coupling of the azide, a solution of CuI (76 mg, 0.12 mmol), TBTA (21 mg, 0.12 mmol) and alkyne 13 (329 mg, 1.2 mmol) in a mixture of DMF (2.5 mL) and THF (2.5 mL) was prepared and reacted with the resin for 48 h at room temperature. After washing with DMF (3 × 1 min × 5 mL), 50 mM aq. EDTA solution (3 × 1 min × 5 mL), DMF (3 × 1 min × 5 mL) and DCM (3 × 1 min × 5 mL), the compound was cleaved by agitating the resin with a mixture of TFA (4.4 mL), TIS (100 µL), H$_2$O (100 µL), m-cresol (200 µL) and thioanisol (200 µL) for 2 h at room temperature. The resin was washed with TFA (1 × 5 min × 5 mL) and the combined cleavage and washing solutions added drop-wise to ice cold diethyl ether (100 mL). The precipitate was collected by centrifugation, dissolved in aq. MeCN and lyophilised to yield the title compound as an off-white powder (468 mg, 0.3 mmol, quant.).

$^1$H-NMR (400 MHz, DMSO-d$_6$) δ [ppm] = 13.09 (s, 2H), 8.37 (s, 4H), 8.29-8.26 (m, 3H), 8.14 (d, J = 8.0 Hz, 1H), 7.91 (s, 2H), 7.80-7.78 (m, 3H), 7.71 (d, J = 8.0 Hz, 1H), 7.65 (t, J = 5.4 Hz, 1H), 4.60-4.48 (m, overlaps with broad H$_2$O peak), 4.33 (t, J = 7.0 Hz, overlaps with broad H$_2$O peak), 4.19-4.13 (m, overlaps with broad H$_2$O peak), 3.64-3.59 (m, 6H), 3.44 (t, J = 6.3 Hz, 2H), 3.27-3.23 (m, 2H), 3.05-3.00 (m, 4H), 2.77-2.48 (m, overlaps with solvent peak), 2.20 (t, J = 7.2 Hz, 4H), 2.04-1.96 (m, 4H), 1.86-1.78 (m, 4H), 1.74-1.63 (br m, 1H), 1.61-1.16 (br m, 9H); HRMS: (m/z) [M + H]$^+$ calcd. for C$_{54}$H$_{83}$N$_{22}$O$_{23}$S$_4$ 1535.4879; found 1535.4868.
Figure IX.1: Analytical HPLC trace of 2 using a Synergi 4 µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min⁻¹ with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 260 nm.

Monovalent targeted IRDye conjugate - 3

To IRDye750 NHS ester (100 µg, 84 nmol) in DMSO (10 µL) and DMF (100 µL) was added AAZ derivative 1 (200 µg, 366 nmol) in DMSO (20 µL) and DIPEA (2 µL, 12 µmol). The mixture was allowed to stand at room temperature for 2 h and then directly purified over reversed-phase HPLC (95% A / 5% B to 40% A / 60% B over 30 min). Fractions containing dye conjugate were identified through their characteristic UV/VIS spectrum (λ_max = 750 nm), pooled, lyophilised and dissolved in DMSO (50 µL) to give a dark green stock solution. Its concentration and the reaction yield were determined by measuring the absorbance at 750 nm (ε_{750} = 260,000 M⁻¹ cm⁻¹) of stock samples diluted 1:200 into PBS pH 7.4 (640 µM, 32 nmol, 38%).

HRMS: (m/z) [M + 4H]⁺ calcd. for C\textsubscript{68}H\textsubscript{92}N\textsubscript{11}O\textsubscript{19}S\textsubscript{6} 1558.4890; found 1558.4844.
**Figure IX.2:** Analytical HPLC trace of 3 using a Synergi 4 µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min⁻¹ with linear gradients of 95% A / 5% B to 40% A / 60% B over 30 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 750 nm.

**Bivalent targeted IRDye conjugate - 4**

To IRDye750 NHS ester (100 µg, 84 nmol) in DMSO (10 µL) and DMF (100 µL) was added 2 (200 µg, 130 nmol) in DMSO (20 µL) and DIPEA (2 µL, 12 µmol). The mixture was allowed to stand at room temperature for 2 h and then directly purified over reversed-phase HPLC (95% A / 5% B to 40% A / 60% B over 30 min). Fractions containing dye conjugate were identified through their characteristic UV/VIS spectrum (λ<sub>max</sub> = 750 nm), pooled, lyophilised and dissolved in DMSO (50 µL) to give a dark green stock solution. Its concentration and the reaction yield were determined by measuring the absorbance at 750 nm (ε<sub>750</sub> = 260,000 M⁻¹ cm⁻¹) of stock samples diluted 1:200 into PBS pH 7.4 (287 µM, 14 nmol, 17%).

HRMS: (m/z) [M + 4H]<sup>+</sup> calcd. for C<sub>103</sub>H<sub>141</sub>N<sub>24</sub>O<sub>36</sub>S<sub>8</sub> 2545.7700; found 2545.7703.
Figure IX.3: Analytical HPLC trace of 4 using a Synergi 4 µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min⁻¹ with linear gradients of 95% A / 5% B to 40% A / 60% B over 30 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 750 nm.

Monovalent untargeted IRDye conjugate - 5

To IRDye750 NHS ester (100 µg, 84 nmol) in DMSO (10 µL) and DMF (100 µL) was added 1 (200 µg, 520 nmol) in DMSO (20 µL) and DIPEA (2 µL, 12 µmol). The mixture was allowed to stand at room temperature for 2 h and then directly purified over reversed-phase HPLC (95% A / 5% B to 40% A / 60% B over 30 min). Fractions containing dye conjugate were identified through their characteristic UV/VIS spectrum ($\lambda_{max} = 750$ nm), pooled, lyophilised and dissolved in DMSO (50 µL) to give a dark green stock solution. Its concentration and the reaction yield were determined by measuring the absorbance at 750 nm ($\varepsilon_{750} = 260,000$ M⁻¹ cm⁻¹) of stock samples diluted 1:200 into PBS pH 7.4 (608 µM, 30 nmol, 36%).

HRMS: (m/z) [M + 4H]⁺ calcd. for C₆₆H₉₈N₇O₁₈S₄ 1396.5220; found 1396.5218.
*Figure IX.4:* Analytical HPLC trace of 5 using a Synergi 4µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min⁻¹ with linear gradients of 95% A / 5% B to 40% A / 60% B over 30 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 750 nm.

**Bivalent untargeted IRDye conjugate - 6**

To IRDye750 NHS ester (100 µg, 84 nmol) in DMSO (10 µL) and DMF (100 µL) was added 10 (200 µg, 165 nmol) in DMSO (20 µL) and DIPEA (2 µL, 12 µmol). The mixture was allowed to stand at room temperature for 2 h and was then directly purified over reversed-phase HPLC (95% A / 5% B to 40% A / 60% B over 30 min). Fractions containing dye conjugate were identified through their characteristic UV/VIS spectrum (λ<sub>max</sub> = 750 nm), pooled, lyophilised and dissolved in DMSO (50 µL) to give a dark green stock solution. Its concentration and the reaction yield were determined by measuring the absorbance at 750 nm (ε<sub>750</sub> = 260,000 M⁻¹ cm⁻¹) of stock samples diluted 1:200 into PBS pH 7.4 (518 µM, 26 nmol, 31%).

HRMS: (m/z) [M + 4H]<sup>+</sup> calcd. for C<sub>99</sub>H<sub>137</sub>N<sub>16</sub>O<sub>34</sub>S<sub>4</sub> 2221.8360; found 2221.8363.
Figure IX.5: Analytical HPLC trace of 6 using a Synergi 4 µm, Polar-RP 150 x 10 mm column at a flow rate of 4 mL min⁻¹ with linear gradients of 95% A / 5% B to 40% A / 60% B over 30 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 750 nm.

**Bivalent targeted DM1 conjugate - 7**

Bivalent targeted linker 11 (20 mg, 13 µmol), TCEP·HCl (7.6 mg, 27 µmol) and DIPEA (2 µL) were dissolved in degassed DMF (500 µL). After 1 h 2,2'-dipyridyldisulphide (11.7 mg, 53 µmol) was added. The mixture was stirred at room temperature for 12 h, diluted with NMP (500 µL) and was added drop wise to ice cold diethyl ether (40 mL). The precipitate was collected by centrifugation, re-dissolved in DMF (200 µL) and NMP (200 µL) and precipitated again with ice cold diethyl ether (40 mL) and dried under vacuum to give the activated disulphide as a white residue (18 mg, 11 µmol, 85%). An aliquot of the activated disulphide (15 mg, 9 µmol) was dissolved in DMF (400 µL) and DM1 free thiol (7 mg, 9 µmol) added. The reaction was allowed to stand at room temperature for 48 h after which the product was recovered by reversed phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min).
Fractions containing the desired product by MS were pooled and lyophilised to yield the title compound as an off white powder (9.5 mg, 4 µmol, 47%).

$^1$H-NMR (500 MHz, DMSO-d$_6$) δ [ppm] = 12.98 (s, 2H), 8.31 (s, 4H), 8.22-8.15 (m, 4H), 8.07 (d, $J$ = 8.2 Hz, 1H), 7.85 (s, 2H), 7.69-7.59 (m, 2H), 7.12 (s, 1H), 6.89 (s, 1H), 6.61-6.52 (m, 3H), 5.92 (br s, 1H), 5.57-5.52 (m, 1H), 5.30-5.29 (m, 1H), 4.52-4.43 (m, 5H), 4.39-4.34 (m, 1H), 4.27 (t, $J$ = 6.9 Hz, 4H), 4.19-4.16 (m, 1H), 4.08-4.03 (m, 1H), 3.92-3.90 (m, 3H), 3.53-2.41 (m, overlap with solvent peak), 2.13-2.12 (m, 4H), 2.04-2.01 (m, 1H), 1.97-1.91 (m, 4H), 1.79-1.73 (m, 4H), 1.67-1.54 (m, 4H), 1.51-1.10 (m, 21H), 0.77 (s, 3H);

HRMS: (m/z) [M + 2H]$^{2+}$ calcd. for C$_{86}$H$_{119}$ClN$_{24}$O$_{33}$S$_6$ 1122.3270; found 1122.3279.

Figure IX.6: Analytical HPLC trace of 7 using a Synergi 4 µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min$^{-1}$ with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 260 nm.

**Bivalent untargeted DM1 conjugate - 8**

Bivalent untargeted linker 12 (20 mg, 17 µmol), TCEP HCl (19 mg, 68 µmol) and DIPEA (10 µL) were dissolved in degassed DMF (1 mL). After 1 h 2,2'-
Dipyridyl disulphide (22 mg, 100 µmol) was added. The mixture was stirred at room temperature for 12 h, diluted with NMP (500 µL) and was added drop wise to ice cold diethyl ether (40 mL). The precipitate was collected by centrifugation, re-dissolved in DMF (200 µL) and NMP (200 µL) and precipitated again with ice cold diethyl ether (40 mL) and dried under vacuum to give the activated disulphide as a white residue (45 mg, product + side products). An aliquot of the residue (15 mg) was dissolved in DMF (400 µL) and DM1 free thiol (7 mg, 9 µmol) was added. The reaction was allowed to stand at room temperature for 48 h after which the product was recovered by reversed phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). Fractions containing the desired product by MS were pooled and lyophilised to yield the title compound as an off white powder (7.4 mg, 3.9 µmol, 42%).

\[ \text{1H-NMR (500 MHz, DMSO-d6) } \delta \text{ [ppm] = 8.22-8.09 (m, 5H), 7.83 (s, 2H), 7.64-7.58 (m, 2H), 7.12 (s, 1H), 6.89 (s, 1H), 6.61-6.52 (m, 3H), 5.93 (s, 1H), 5.55 (dd, } J = 9.1, 14.8 \text{ Hz, 1H), 5.32-5.28 (m, 1H), 4.56-4.43 (m, 6H), 4.27 (t, } J = 6.85 \text{ Hz, 4H), 4.20-4.17 (m, 1H), 4.05 (t, } J = 12.2 \text{ Hz, 1H), 3.91 (s, 3H), 3.49-2.41 (m, overlap with solvent peak), 2.25 (t, } J = 7.4 \text{ Hz, 4H), 2.15 (m, 4H), 2.04-2.02 (br m, 1H), 1.80-1.73 (m, 8H), 1.62-1.10 \text{ m, 24H), 0.77 (s, 3H); HRMS: (m/z) } [M + H]^+ \text{ calcd. for C}_{82}H_{116}ClN_{16}O_{31}S_{2} 1919.7117; \text{ found 1919.7098.} \]

**Figure IX.7:** Analytical HPLC trace of 8 using a Synergi 4 µm, Polar-RP 150 x 10 mm column at a flow rate of 4 mL min⁻¹ with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 260 nm.

*Monovalent linker - 9*
Commercially available polystyrene Wang p-nitrophenyl carbonate resin (250 mg, 0.15 mmol) was swollen in DMF (5 mL for 5 min) and reacted with a solution of 2,2'- (ethane-1,2-diylbis(oxy))diethanamine (250 µL), DIPEA (500 µL) and DMAP (2.5 mg) in DMF (4.5 mL) for 12 h at room temperature under shaking. The resin was washed with DMF (3× 5 mL for 1 min), MeOH (3× 5 mL for 1 min) and again DMF (3× 5 mL for 1 min). A solution of 5-azido valeric acid (65 mg, 0.45 mmol), HATU (171 mg, 0.45 mmol) and DIPEA (148 µL, 0.9 mmol) was prepared and immediately reacted with the resin for 1 h at room temperature under shaking. After washing with DMF (6 × 1 min × 5 mL) a solution of Cul (2.9 mg, 0.015 mmol), TBTA (8 mg, 0.015 mmol) and 5-hexynoic acid (51 mg, 50 µL, 0.45 mmol) in a mixture of DMF (1 mL) and THF (1 mL) was prepared and reacted with the resin for 24 h at room temperature. After washing with DMF (3 × 1 min × 5 mL), 50 mM aq. EDTA solution (3 × 1 min × 5 mL), DMF (3 × 1 min × 5 mL) and DCM (3 × 1 min × 5 mL), the compound was cleaved by agitating the resin with a mixture of TFA (2.2 mL), TIS (50 µL), H2O (50 µL), m-cresol (100 µL) and thioanisol (100 µL) for 2 h at room temperature. The resin was washed with TFA (1 × 5 min × 2.5 mL) and the combined cleavage and washing solutions added drop-wise to ice cold diethyl ether (100 mL). The precipitate was collected by centrifugation and the product purified by reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). After lyophilisation the title compound was collected as a white powder (21 mg, 54 µmol, 36%).

1H-NMR (400 MHz, DMSO-d6) δ [ppm] = 7.90-7.86 (m, 5H), 4.29 (t, J = 7.0 Hz, 2H), 3.60-3.51 (m, 6H), 3.40 (t, J = 6.1 Hz, 2H), 3.20 (m, 2H), 3.00-2.96 (m, 2H), 2.62 (t, J = 7.6 Hz, 2H), 2.26 (t, J = 7.4 Hz, 2H), 2.10 (t, J = 7.4 Hz, 2H), 1.85-1.74 (m, 4H), 1.46-1.42 (m, 2H); 13C-NMR (125 MHz, DMSO-d6) δ [ppm] = 174.8, 172.4, 146.7, 122.3, 70.1, 69.8, 69.5, 67.2, 49.4, 39.0, 38.9, 35.0, 33.6, 29.8, 24.9, 24.8, 22.7; HRMS: (m/z) [M + H]⁺ calcd. for C117H32N8O3 386.2398; found 386.2403.

Bivalent linker - 10
Commerciaally available polystyrene Wang ρ-nitrophenyl carbonate resin (500 mg, 0.3 mmol) was swollen in DMF (5 mL for 5 min) and reacted with a solution of 2,2'(ethane-1,2-diylbis(oxy))diethanamine (500 µL), DIPEA (500 µL) and DMAP (5 mg) in DMF (4 mL) for 12 h at room temperature under shaking. The resin was washed with DMF (3× 5 mL for 1 min), MeOH (3× 5 mL for 1 min) and again DMF (3× 5 mL for 1 min). A solution of Fmoc-Lys(Fmoc)-OH (532 mg, 0.9 mmol), HBTU (341 mg, 0.9 mmol), HOBt (138 mg, 0.9 mmol) and DIPEA (298 µL, 1.8 mmol) was prepared and immediately reacted with the resin for 1 h at room temperature under shaking. After washing with DMF (6× 1 min × 5 mL) the Fmoc group was removed with 20 % piperidine in DMF (1× 1 min × 5 min and 2× 10 min × 5 mL) and the resin washed with DMF (6× 1 min × 5 mL) before the next coupling step was initiated. In the following, the peptide was extended with Fmoc-Asp(OtBu)-OH twice followed by 5-azido-valerate. For this purpose, a solution of acid (1.2 mmol), HATU (465 mg, 1.2 mmol) and DIPEA (397 µL, 2.4 mmol) was prepared in DMF (5 mL) and reacted with the resin for 1 h at room temperature under gentle agitation. Each coupling was followed by a washing step with DMF (6× 1 min × 5 mL) and Fmoc deprotection as described above. After coupling of the azide, a solution of Cul (76 mg, 0.12 mmol), TBTA (21 mg, 0.12 mmol) and 5-hexyonic acid (440 µL, 1.2 mmol) in a mixture of DMF (2.5 mL) and THF (2.5 mL) was prepared and reacted with the resin for 48 h at room temperature. After washing with DMF (3× 1 min × 5 mL), 50 mM aq. EDTA solution (3× 1 min × 5 mL), DMF (3× 1 min × 5 mL) and DCM (3× 1 min × 5 mL), the compound was cleaved by agitating the resin with a mixture of TFA (4.4 mL), TIS (100 µL), H₂O (100 µL), m-cresol (200 µL) and thioanisol (200 µL) for 2 h at room temperature. The resin was washed with TFA (1× 5 min × 5 mL) and the combined cleavage and washing solutions added drop-wise to ice cold diethyl ether (100 mL). The precipitate was collected by centrifugation and the product purified by reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). After lyophilisation the title compound was collected as a white powder (64 mg, 53 µmol 17%).

\[ ^1H-NMR (400 MHz, DMSO-d_6) \delta [ppm] = 8.25-8.22 (m, 3H), 8.09 (d, J = 8.1 Hz, 1H), 7.85 (s, 2H), 7.78-7.73 (br m, 3H), 7.66 (d, J = 7.9 Hz, 1H), 7.59 (t, J = 5.3 Hz, 1H), 4.55-4.44 (m, 4H), 4.29 (t, J = 7.0 Hz, 4H), 4.14-4.09 (m, 2H), 3.60-3.55 (m, 6H), 3.40 (t, J = 6.2 Hz, 2H), 3.22-3.19 (m, 2H), 3.01-2.92 (m, 4H), 2.73-2.44 (m, overlap with solvent peak), 2.26 (t, J = 7.4 Hz, 4H), 2.15 (t, J = 7.2 Hz, 4H), 1.85-1.74 (m, 7H),\]
1.70-1.60 (br m, 1H), 1.55-1.14 (br m, 9H); HRMS: (m/z) [M + H]$^+$ calcd. for C$_{50}$H$_{79}$N$_{14}$O$_{21}$ 1211.5539; found 1211.5515.

**Figure IX.8**: Analytical HPLC trace of 10 using a Synergi 4 µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min$^{-1}$ with linear gradients of 95% A / 5% B to 50% A / 50% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 210 nm.

**Bivalent targeted Cys ligand derivative - 11**

Commercially available pre-loaded Fmoc-Cys(Trt) on Tentagel resin (500 mg, 0.415 mmol, RAPP Polymere) was swollen in DMF (3 × 5 min × 5 mL), the Fmoc group removed with 20 % piperidine in DMF (1 × 1 min × 5 mL and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL). A solution of Fmoc-Lys(Fmoc)-OH (736 mg, 1.25 mmol), HBTU (472 mg, 1.25 mmol), HOBt (191 mg, 1.25 mmol) and DIPEA (412 µL, 2.5 mmol) was prepared and immediately reacted with the resin for 1 h at room temperature under shaking. After washing with DMF (6 × 1 min × 5 mL) the Fmoc group was removed with 20 % piperidine in DMF (1 × 1 min × 5 min and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL) before the next coupling step was initiated. In the following, the peptide was extended with Fmoc-Asp(OtBu)-OH twice followed by 5-azido-valerate. For this purpose, a solution of acid (1.7 mmol), HATU (643 mg, 1.7 mmol) and DIPEA (549 µL, 3.3 mmol) was prepared in DMF (5 mL) and reacted with the resin for 1 h at room temperature under gentle
agitation. Each coupling was followed by a washing step with DMF (6 × 1 min × 5 mL) and Fmoc deprotection as described above. After coupling of the azide, a solution of CuI (106 mg, 0.17 mmol), TBTA (29 mg, 0.17 mmol) and alkyne 13 (455 mg, 1.7 mmol) in a mixture of DMF (2.5 mL) and THF (2.5 mL) was prepared and reacted with the resin for 48 h at room temperature. After washing with DMF (3 × 1 min × 5 mL), 50 mM aq. EDTA solution (3 × 1 min × 5 mL), DMF (3 × 1 min × 5 mL) and DCM (3 × 1 min × 5 mL), the compound was cleaved by agitating the resin with a mixture of TFA (4.4 mL), TIS (100 µL), H₂O (100 µL), m-cresol (200 µL) and thioanisol (200 µL) for 2 h at room temperature. The resin was washed with TFA (1 × 5 min × 5 mL) and the combined cleavage and washing solutions added drop-wise to ice cold diethyl ether (100 mL). The precipitate was collected by centrifugation and the product purified by reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). After lyophilisation the title compound was collected as a white powder (68 mg, 45 µmol, 10%).

¹H-NMR (400 MHz, DMSO-d₆) δ [ppm] = 13.01 (s, 2H), 8.32 (s, 4H), 8.21 (t, J = 7.5 Hz, 3H), 8.09 (d, J = 8.1 Hz, 1H), 8.05 (d, J = 7.9 Hz, 1H), 7.87 (s, 2H), 7.74 (d, J = 7.84 Hz, 1H), 7.61 (t, J = 5.4 Hz, 1H), 4.55-4.45 (m, overlap with broad water peak), 4.40-4.34 (m, overlap with broad water peak), 4.29 (t, J = 7.0 Hz, overlap with broad water peak), 4.24-4.22 (m, overlap with broad water peak), 3.07-2.94 (br m, 2H), 2.90-2.41 (m, overlap with solvent peak), 2.15 (t, J = 7.1 Hz, 4H), 1.99-1.92 (m, 4H), 1.82-1.74 (m, 4H), 1.71-1.24 (br m, 10H); HRMS: (m/z) [M + H]⁺ calcd. for C₅₁H₇₄N₂₁O₂₂S₅ 1508.3864; found 1508.3861.

**Figure IX.9:** Analytical HPLC trace of 11 using a Synergi 4 µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min⁻¹ with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 260 nm.
Commercially available pre-loaded Fmoc-Cys(Trt) on Tentagel resin (500 mg, 0.415 mmol, RAPP Polymere) was swollen in DMF (3 × 5 min × 5 mL), the Fmoc group removed with 20 % piperidine in DMF (1 × 1 min × 5 mL and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL). A solution of Fmoc-Lys(Fmoc)-OH (736 mg, 1.25 mmol), HBTU (472 mg, 1.25 mmol), HOBt (191 mg, 1.25 mmol) and DIPEA (412 µL, 2.5 mmol) was prepared and immediately reacted with the resin for 1 h at room temperature under shaking. After washing with DMF (6 × 1 min × 5 mL) the Fmoc group was removed with 20 % piperidine in DMF (1 × 1 min × 5 min and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL) before the next coupling step was initiated. In the following, the peptide was extended with Fmoc-Asp(OtBu)-OH twice followed by 5-azido-valerate. For this purpose, a solution of acid (1.7 mmol), HATU (643 mg, 1.7 mmol) and DIPEA (549 µL, 3.3 mmol) was prepared in DMF (5 mL) and reacted with the resin for 1 h at room temperature under gentle agitation. Each coupling was followed by a washing step with DMF (6 × 1 min × 5 mL) and Fmoc deprotection as described above. After coupling of the azide, a solution of Cul (106 mg, 0.17 mmol), TBTA (29 mg, 0.17 mmol and 5-hexyonic acid (609 µL, 1.7 mmol) in a mixture of DMF (2.5 mL) and THF (2.5 mL) was prepared and reacted with the resin for 48 h at room temperature. After washing with DMF (3 × 1 min × 5 mL), 50 mM aq. EDTA solution (3 × 1 min × 5 mL), DMF (3 × 1 min × 5 mL) and DCM (3 × 1 min × 5 mL), the compound was cleaved by agitating the resin with a mixture of TFA (4.4 mL), TIS (100 µL), H₂O (100 µL), m-cresol (200 µL) and thioanisol (200 µL) for 2 h at room temperature. The resin was washed with TFA (1 × 5 min × 5 mL) and the combined cleavage and washing solutions added drop-wise to ice cold diethyl ether (100 mL). The precipitate was collected by centrifugation and the product purified by reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). After lyophilisation the title compound was collected as a white powder (147 mg, 0.12 mmol, 30%).
$^1$H-NMR (500 MHz, DMSO-d$_6$) δ [ppm] = 8.22-8.19 (m, 3H), 8.08 (d, $J$ = 8.9 Hz, 1H), 8.02 (d, $J$ = 7.8 Hz, 1H), 7.83 (s, 2H), 7.72 (d, $J$ = 7.8 Hz, 1H), 7.59-7.56 (m, 1H), 4.56-4.43 (m, 3H), 4.37-4.34 (m, 1H), 4.27-4.20 (m, 4H), 3.03-2.92 (m, 2H), 2.87-2.39 (m, overlap with solvent peak), 2.25 (t, $J$ = 7.35 Hz, 4H), 2.13 (t, $J$ = 7.0 Hz, 4H), 1.83-1.21 (br m, 16H); HRMS: (m/z) [M + H]$^+$ calcd. for C$_{47}$H$_{70}$N$_{13}$O$_{21}$S 1184.4524; found 1184.4508.

**Figure IX.10:** Analytical HPLC trace of 12 using a Synergi 4 μm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min$^{-1}$ with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 210 nm.

**Acetazolamide derivative - 13**

![Acetazolamide derivative - 13](image)

Compound 13 was prepared according to previously described methods.$^{[59]}$
2.3. NMR Spectra

500 MHz proton spectrum of 1 in DMSO-d6

125 MHz carbonspectrum of 1 in DMSO-d6
400 MHz proton spectrum of 2 in DMSO-d6

500 MHz proton spectrum of 7 in DMSO-d6
125 MHz carbon spectrum of 9 in DMSO-d6

400 MHz proton spectrum of 10 in DMSO-d6
400 MHz proton spectrum of 11 in DMSO-d6

400 MHz proton spectrum of 12 in DMSO-d6
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