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Drug Delivery

Small Targeted Cytotoxics: Current State and Promises from DNA-Encoded Chemical Libraries

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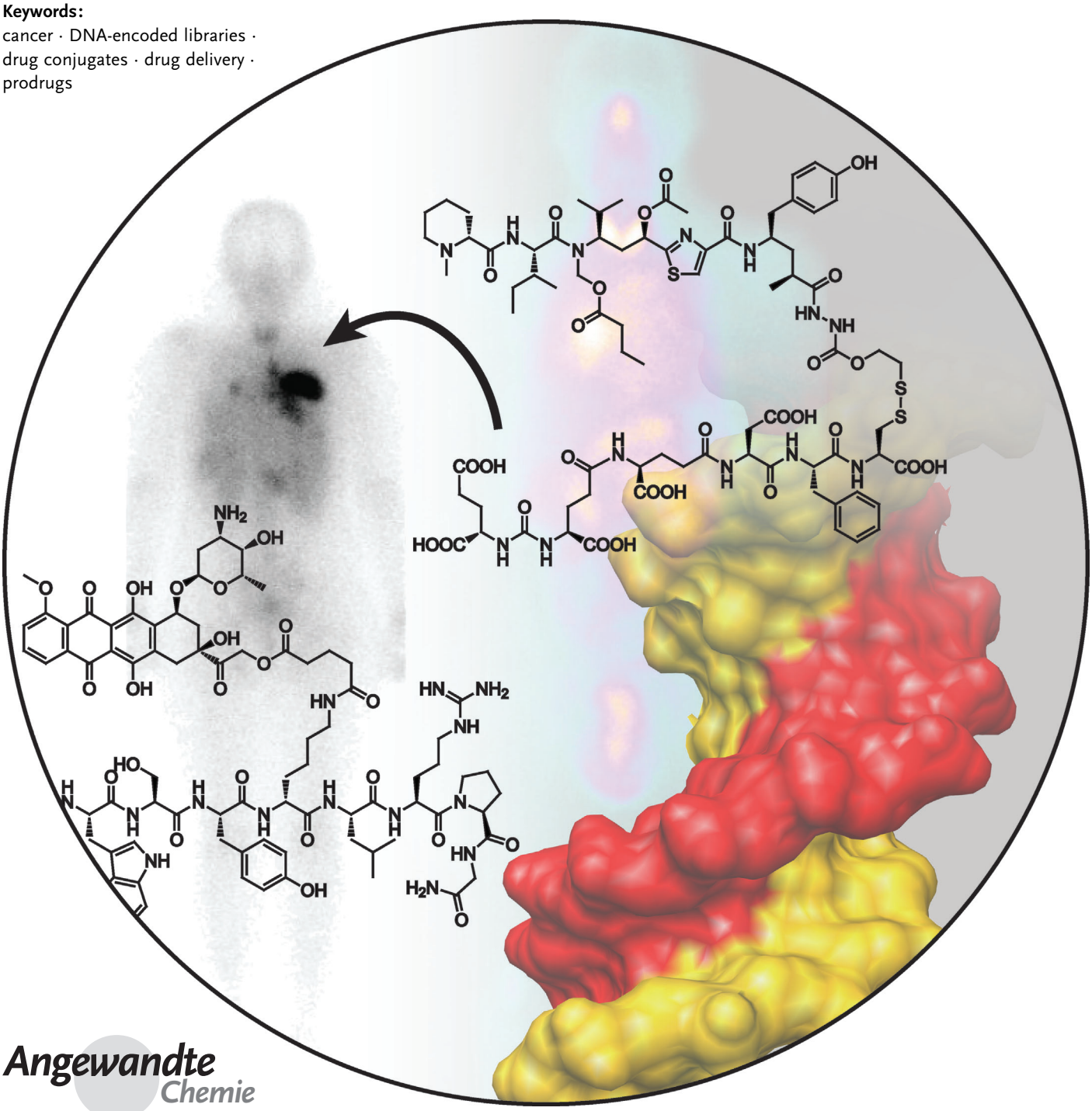

The targeted delivery of potent cytotoxic agents has emerged as a promising strategy for the treatment of cancer and other serious conditions. Traditionally, antibodies against markers of disease have been used as drug-delivery vehicles. More recently, lower molecular weight ligands have been proposed for the generation of a novel class of targeted cytotoxics with improved properties. Advances in this field crucially rely on efficient methods for the identification and optimization of organic molecules capable of high-affinity binding and selective recognition of target proteins. The advent of DNA-encoded chemical libraries allows the construction and screening of compound collections of unprecedented size. In this Review, we survey developments in the field of small ligand-based targeted cytotoxics and show how innovative library technologies will help develop the drugs of the future.

From the Contents

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,[1] lupus,[2] multiple sclerosis).[3] 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[4] and, thus, have an impact on normal adult tissues, which constantly regenerate (such as epithelial structures in the gastrointestinal tract and in the skin, hair growth, haematopoiesis).

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 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).[5] Similar findings have been reported for other anticancer drugs, including ^{18}F-labeled 5-fluorouracil,[6] ^{3}H-labeled paclitaxel,[7,8] and Pt^{II} derivatives (Figure 1a,b).[9]

The accumulation of small organic drugs in solid tumor masses is hindered by the high interstitial pressure at the tumor site,[10] irregular vasculature,[11,12] and by the fact that tumor cells frequently overexpress multidrug resistance proteins.[13]

Consequently, there is 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.

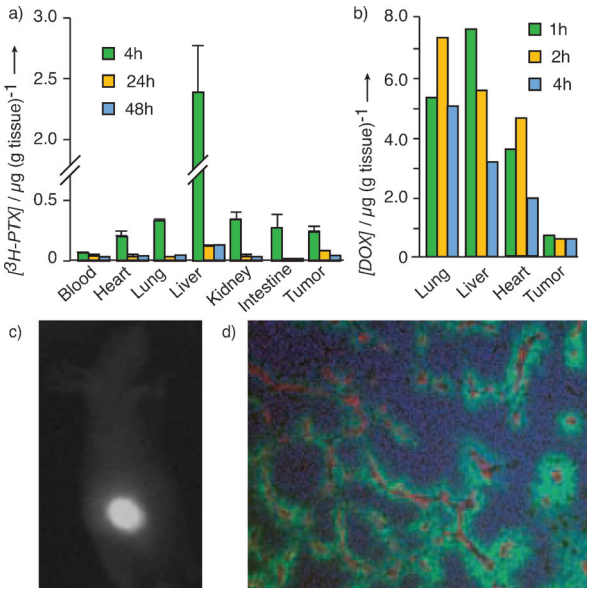

Figure 1. a) Tissue distribution of ^{3}H-labeled paclitaxel (^{3}H-PTX) in tumor-bearing mice.[8] b) Tissue distribution of doxorubicin (DOX) in tumor-bearing mice.[5] c) Macroscopic accumulation of an antibody against a splice isoform of tenascin C in a solid tumor. d) Trapping of fluorescently labeled trastuzumab (green) outside tumor blood vessels (red). (Reprinted by permission from the American Association for Cancer Research.[34])

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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 (Figure 2a).[14,15] Nowadays, human monoclonal antibodies can be raised against virtually any target protein of interest,[16] and certain antibodies have exhibited a striking ability to selectively localize in solid tumors (Figure 1c)[17–21] and at other sites of disease, such as in rheumatoid arthritis,[22] endometriosis,[23] and atherosclerosis.[24]

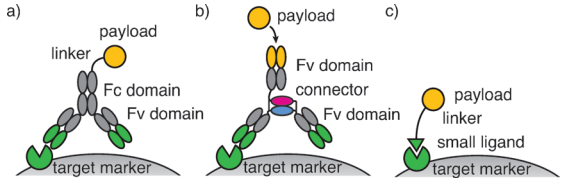

Figure 2. a) Direct delivery of a payload (yellow) with an armed antibody specific for a marker protein of disease (green). b) Pretargeting with a bispecific antibody binding a marker of the disease (green) and a payload (yellow) administered after clearance of the antibody from healthy tissue. c) Direct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

Since cytotoxic drugs are usually active inside the cell, it has long been postulated that antibodies which are capable of selective internalization into the target cell and equipped with cleavable linkers would be ideally suited for the targeted delivery of cytotoxic agents.[14,15] Recently, it has, however, become clear that the internalization of antibodies is not a strict requirement for targeted drug delivery, as antibody–drug conjugates specific to extracellular matrix proteins can mediate a potent antitumor effect if equipped with cleavable disulfide-based linkers.[25,26]

It remains open which classes of target antigens and which chemical coupling strategies may be preferable for the development of therapeutic antibody–drug conjugates. However, 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,[27–29] for the treatment of Hodgkin lymphoma. Furthermore, more than 30 antibody–drug conjugates are currently in clinical testing, primarily for applications in oncology.[14]

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 in vivo implementation of the technology can be much more difficult. There are a number of limitations to 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, thus reflecting the need for the separate industrial manufacturing of clinical-grade antibodies by fermentation technology, of clinical-grade drugs, 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 in vivo and which may be easier to produce (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.[30] Furthermore, the "antigen barrier" hypothesis has been postulated as a main obstacle to the penetration of a homogeneous antibody into the tumor mass.[31–33] Indeed, large macromolecules (including antibodies) do not extravasate (i.e. exit blood vessels) and diffuse efficiently into tissue. The few antibody molecules which reach the abluminal side of blood vessels are immediately trapped by antigens located on perivascular tumor cells, thus preventing the targeting of tumor cells distant from blood vessels (Figure 1d).[34]

The tumor-targeting performance of many monoclonal antibodies has been studied extensively in tumor-bearing mice by 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.[35–38] Seduced by the preferential

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Jörg Scheuermann studied Chemistry at the Ruprecht-Karls-University Heidelberg (Germany) and ETH Zurich (Switzerland). During his PhD in the group of Prof. Dario Neri at the ETH Zurich he worked on the identification of novel binding molecules to markers of angiogenesis. Together with Prof. Neri he later co-developed the DNA-encoded self-assembling chemical (ESAC) libraries. Currently, he is working on his habilitation in the field of antibody–drug conjugates and DNA-encoded chemical libraries and their application in drug discovery and development.

accumulation of antibodies at the site of the 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 in vivo: virtually all of them accumulate (at least transiently) in excretory organs (liver for intact antibodies, kidneys for small antibody fragments).[39] Indeed, variations in off-target toxicity associated with clearance may make the crucial difference in the development and registration of a cytotoxic agent (e.g. approval of oxaliplatin because of very low kidney toxicity).[40]

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.[20,21,39] At the other extreme, small antibody formats (such as scFv fragments) are cleared more rapidly from the circulation, but also exhibit reduced tumor uptake.[39,41]

Finally, antibodies can be immunogenic, even when they are humanized or fully human.[42] The development of an undesired antibody reaction may not only cause hypersensitivity in patients, but also neutralize the therapeutic effect of the antibody drugs and alter the 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 accumulate preferentially 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 can be visualized by nuclear staining with Hoechst 33342 at different time points after intravenous administration.[43]

The potential benefits associated with the use of small tumor-targeting agents are best exemplified by the analysis of "pretargeting" strategies, where bispecific antibodies (capable of recognizing a tumor-associated antigen and a metal chelator) are allowed to localize at tumors and clear from the circulation, before being targeted by a low-molecular-weight radiometal–chelator complex (Figure 2b).[44,45]

Dario Neri studied Chemistry at the Scuola Normale Superiore of Pisa (Italy) and at the ETH Zurich (Switzerland). After postdoctoral research at the Medical Research Council, Cambridge (UK), in 1996 he returned to the ETH Zürich as a professor. His research focuses on strategies for the targeted delivery of therapeutic effectors to sites of disease and the development of DNA-encoded chemical libraries. He is cofounder of Philogen, a Swiss–Italian biotech company, which has brought several antibody drugs into clinical development programs.

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 pretargeting implementation of the same antibodies showed that the maximum concentrations of the radiometal–chelator complexes in the tumor (percent injected dose per gram, % ID g^{-1}) 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 pretargeting approach, with strikingly good tumor to organ ratios (>10:1) already one hour after injection.[44]

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, may overcome some of the limitations of antibody–drug conjugates. Indeed, small-ligand-targeted drugs (Figure 2c) are expected to rapidly reach their target in vivo, to be non-immunogenic, and to be amenable to chemical synthesis.

2. Natural Small Delivery Vehicles and Their Analogues

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[46–49] and vitamins.[50,51] These ligands often display high affinities to their cognate receptors and undergo a rapid receptor-mediated endocytosis after binding. Substrate analogues have also been investigated as potent binders for tumor-associated enzymes, such as prostate-specific membrane antigen.[52,53]

The binding of the ligand to the target cell results in the ligand–receptor complex folding into a vesicle, which subsequently fuses with the early endosome (Figure 3). A payload may be released at this stage by cleavage of the disulfide bond or the lowered pH value. Alternatively, the targeted cytotoxic agent may proceed to the lysosome, where drug release may occur through the catalytic activity of

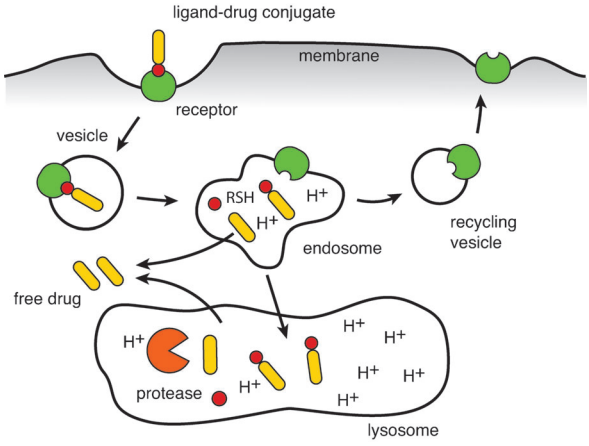


Figure 3. Mechanism of uptake and activation of ligand-targeted drug conjugates.

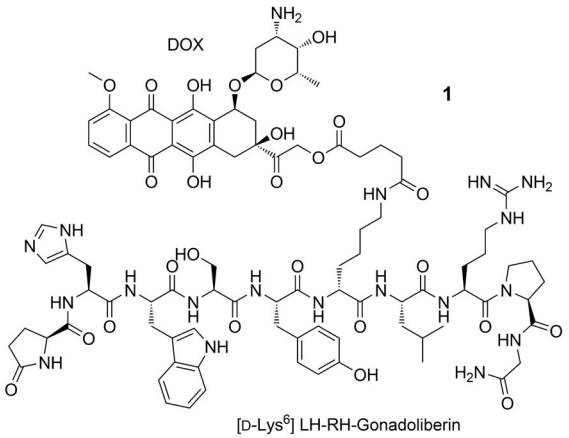
proteases and esterases or through a further reduction of the pH value. Finally, the drug needs to escape the endosome or lysosome by passive diffusion in order to act on cytosolic or nuclear targets.[54,55]

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.[56,57]

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.[56] Several radiolabeled peptides have now reached the clinic and show very encouraging performance as imaging or radiotherapeutic agents, thus providing validation for the peptide-targeting approach.[58,59]

Pioneering work in the area of peptide–drug conjugates from Schally and co-workers revealed that derivatives of the luteinizing hormone-releasing hormone (LH-RH) could be used for the delivery of the cytotoxic agents cisplatin and [*trans*-bis(salicylaldoximato)copper(II)].[60] Subsequently, conjugates were prepared of somatostatin, bombesin, and LH-RH analogues as the targeting ligands and doxorubicin or 2-pyrrolinodoxorubicin as the active drug. Usually, a lysine side chain or the terminal amine of the peptide and the primary alcohol of the drug were connected through a glutaryl spacer (Scheme 1). The constructs typically exhibited good receptor-binding affinities in vitro (i.e. K_D values in the low nanomolar range, as estimated from competition experiments against radiolabeled peptides using receptor-positive whole cells or membrane preparations), yet cytotoxicity was found not to be increased compared to the unmodified drug.[61–63] In vivo, such conjugates exhibited a more potent retardation of tumor growth than the cytotoxic payload alone, and showed lower toxicity in several cancer models.[64–68] 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.[64,68–70] Despite the potentially stable nature of the linker, no evidence was provided about the efficiency, mechanism, or 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.[71]



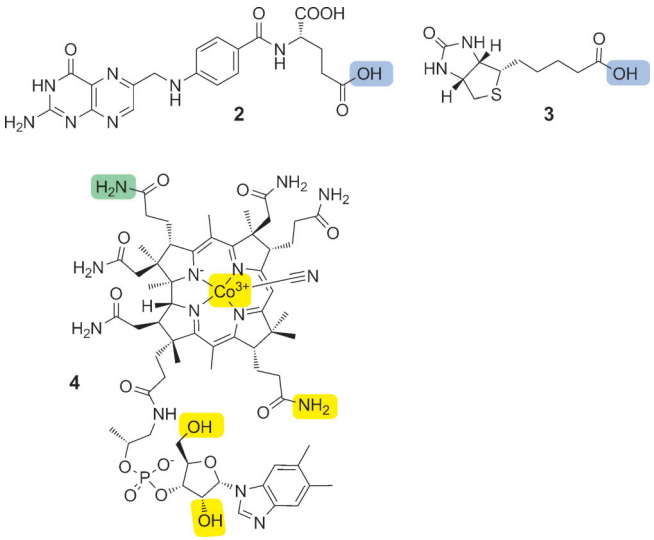
Scheme 1. The peptide-targeted drug conjugate AN-152 (**1**) consisting of the [D-Lys^6]-luteinizing hormone-releasing hormone (LH-RH) as the targeting moiety and doxorubicin (DOX) as the cytotoxic payload linked through a glutaryl spacer.[62]

Related peptide ligands have also been tested in vivo as conjugates with other drugs. For example, Kompella and co-workers reported a conjugate of docetaxel glutaryl ester linked to a serine residue of deslorelin, a very potent LH-RH agonist.[72] 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 ± 0.06) nM, respectively) over docetaxel ($IC_{50} = (4.47 \pm 0.93)$ and (6.26 ± 1.24) nM, respectively) in vitro and also showed improved retardation of tumor growth in PC-3 xenografts in vivo. Coy and co-workers coupled the cytotoxic compound camptothecin to analogues of somatostatin and bombesin through carbamate linkers.[73,74] 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 in vitro ($IC_{50} = 64.1$ nM–4.83 μM for the conjugates compared to 3.10–259 nM for the free drug), possibly because of the high stability of the linker. 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 μM) than to free camptothecin ($IC_{50} = 3.08$ μM) in vitro, and tumor growth was also slowed down in vivo. In the case of bombesin, the conjugate CPT-L2-BA3 exhibited cytotoxicity against a range of human cancer cell lines ($IC_{50} = 33$ nM–2.7 μM) 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 the 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 2), because of their very rapid cell-division cycles.[75] 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.[50,54,55,76]

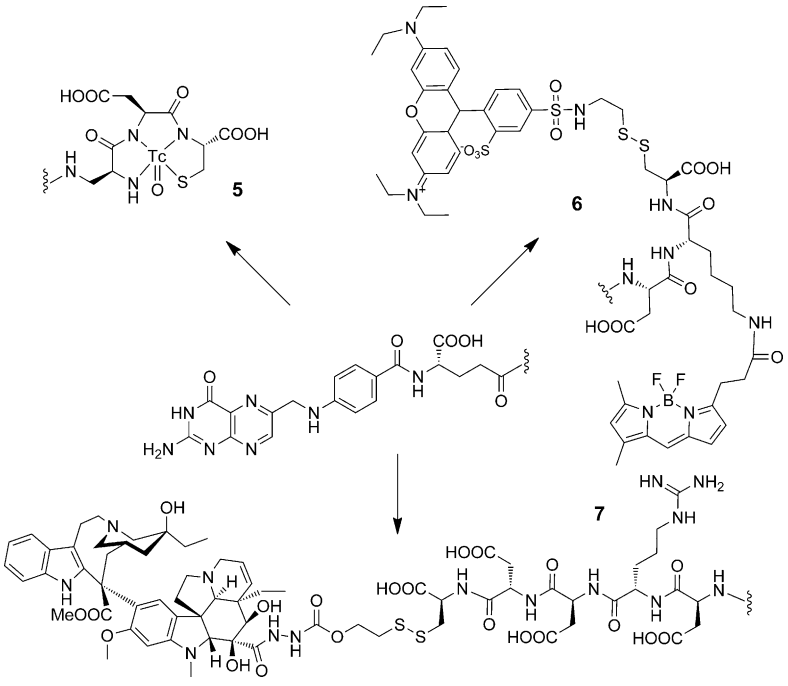


Scheme 2. Vitamins commonly used for drug-delivery applications. The functional groups on folate (**2**) and biotin (**3**) amenable to payload attachment are shaded in blue.[50,51,55] Payloads attached to the yellow shaded groups on cobalamin (**4**) have been shown to maintain full binding to all cobalamin-trafficking proteins, whilst attachment of the payload through the group shaded in green allows the generation of transcobalamin I selective constructs.[76]

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[50,77]), which is abundantly overexpressed in many malignant tumors (e.g. up to 80 pmol (mg membrane protein)$^{-1}$ in KB cells[78]) and can rapidly shuttle conjugates into the cell (e.g. at a rate of up to 3.4×10^5 molecules cell^{-1} h^{-1} in KB cells[79]). Radiometal chelators (e.g. **5**) and various fluorophores (e.g. **6**) have been used as folate conjugates in imaging studies (Scheme 3).[80–86] The folate-tethered protein toxin pseudomonas exotoxin (PE38, cytotoxicity down to $IC_{50} = 20$ pM for a disulfide-bridged conjugate in FDHeLa cells)[87] and conjugates with the natural product cytotoxics camptothecin ($IC_{50} = 10$ nM in KB cells),[88] desacetylvinblastine ($IC_{50} = 11$ nM in KB cells),[89–91] the maytansinoid DM1 ($IC_{50} = 16$ nM in KB cells),[78] tubulysins A and B ($IC_{50} = 7$ nM in KB cells),[92,93] and mitomycin C ($IC_{50} = 5$ nM in KB cells)[94,95] have been investigated as targeted therapeutics. Folate-coated liposomal drug carriers have also been reported,[96–98] as has hapten-based immunotherapy.[99] In the latter case 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 desacetylvinblastine as payloads have recently been proposed.[100] As a result of these research efforts, three different folate–drug conjugates and one imaging agent are currently being investigated in clinical trials.[101,102] In particular, EC145 (**7**; $IC_{50} = 11$ nM in KB cells) has reached phase III testing in patients with ovarian cancer. The molecules described above have recently been covered in a number of excellent reviews.[50,55,103] For this reason, we will summarize here only the findings which are most relevant for our discussion.

Folate-based radiopharmaceuticals rapidly accumulate at the tumor site and quickly clear from FR-negative tissue. The conjugate formed from ^{111}indium-diethylenetriamine pentaacetic acid (DTPA) and folate, previously shown to be taken up in a FR-dependent manner in vitro, already attained maximum tumor accumulation 30 min after injection in xenograft mouse models.[83] Absolute tumor uptake values were high (up to 10% ID g^{-1}) and essentially stable over a period of 24 h. Efficient clearance from FR-negative tissue occurred in less than 1 h, with the exception of the 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 uptake of the active conjugate.[104] It is not known whether the radiolabeling method also influenced uptake values in the kidney, but similar results were reported for ^{99m}Tc-labeled folate conjugates.[81,84]

The use of fluorescently labeled folate analogues was crucial for the demonstration of a rapid extravasation of this class of small organic tumor-targeting agents. Folate–rhod-



Scheme 3. ^{99m}Tc-labeled folate conjugate EC20 (**5**)[86] and disulfide FRET probe **6**[77] for imaging applications, as well as desacetylvinblastine–folate conjugate EC145 (**7**)[89,91] for targeted drug delivery to folate–receptor-positive tumors.

amine conjugates were studied in vivo by using multiphoton microscopy, and revealed that these agents rapidly exit blood vessels and that folate receptors on the tumor could be saturated within 5 minutes after intravenous administration. Periorbital and intraperitoneal administration gave rise to saturation times of < 30 min and < 100 min. It was also shown in the same study 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}).[105] These results make a strong case for the use of small organic ligands if rapid clearance and rapid accumulation at the site of the disease are needed.

The discovery that activated macrophages could take up folate (**2**) in an FR-dependent manner[106] opened the possibility to image sites of inflammation[107] 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.[108] Similar findings have previously been reported for the uptake of biotin (**3**) at sites of infection.[109]

As a first attempt, fluorescein-based hapten immunotherapy showed promising results in the treatment of animal models of arthritis.[110] Recently, a folate–aminopterin conjugate was reported which shows high activity against the adjuvant-induced arthritis model in rats.[111] A conjugate of didemnin B and folate has been shown to exhibit potent ($IC_{50} = 13$ nM) cytotoxicity and anti-TNF-α activity in RAW264.7 macrophages.[112]

Initial evidence for the importance of the chemical nature of the linker connecting folate (**2**) and its payloads came from studies with conjugates of folate and pseudomonas exotoxin (PE38).[87] The observed cytotoxicity with a reducibly cleavable disulfide bond as the linkage was four orders of magnitude higher than that of a conjugate based on a stable thioether bond. The release of payloads from folate-based conjugates by cleavage of the disulfide was further investigated directly using the folate Förster resonance energy transfer (FRET) probe **6**.[77] It was demonstrated that the probe never reached the lysosome. In fact, cleavage of the disulfide was already observed in the endosome, from which the folate–FR complex seemed to be directly recycled to the cell surface. It was concluded from fluorescence-based pH measurements in endosomal compartments that the environment was not sufficiently acidic to cleave acid-labile drug–folate linkages.[113] The in vivo comparison of two folate conjugates with desacetylvinblastine conjugated to folate (**2**) either through a self-immolative disulfide-based linker or an acid-labile hydrazone linkage further underpinned these findings. Whilst the disulfide-based conjugate showed remarkable activity, the hydrazone-based conjugate only exhibited moderate retardation of tumor growth.[91] 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.[114]

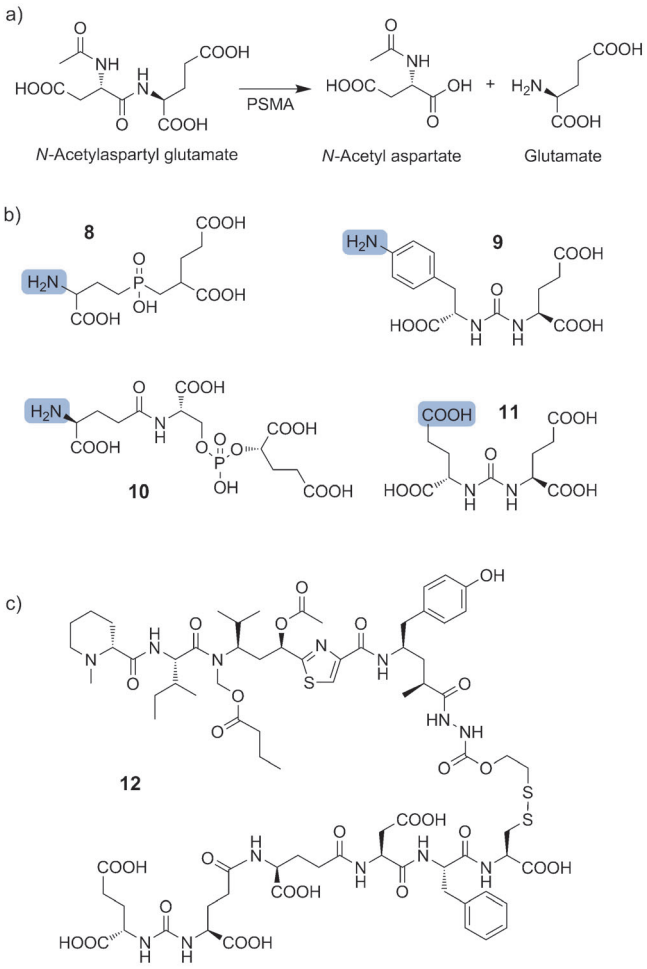
Besides folate (**2**), cobalamin (vitamin B_{12}, **4**), which is essential for thymidine biosynthesis, has also been actively investigated as a ligand for the delivery of cytotoxic drugs into solid tumors.[76] Indeed, ^{57}Co-vitamin B_{12} was tested for imaging applications in murine models of cancer. The translation of this approach into the clinic was hampered by the high uptake in the liver, pancreas, and kidneys.[115] Nevertheless, cobalamin conjugates with chelators for radiometals such as ^{99m}Tc and ^{111}In, which are more suitable for imaging in humans, were prepared,[116,117] as well as several conjugates of derivatives of cisplatin[118] and an acid-cleavable conjugate to the microtubule-stabilizing agent colchicine.[119] In all of these approaches, the therapeutic payloads were attached at modification sites (Scheme 2) which were known to allow constructs to retain their binding affinity for all physiologically important cobalamin carriers, namely transcobalamin I (TCI, haptocorrin), intrinsic factor (IF), and transcobalamin II (TCII). Only later was it realized that whilst TCII and the corresponding TCII receptor were responsible for the uptake of cobalamin from the bloodstream into normal tissues, membrane-bound TCI was highly expressed in certain types of tumors. Cobalamin conjugates which did not bind TCII but retained their binding affinity for TCI were thus synthesized and showed improved targeting properties in vivo.[120]

Similar to the situation encountered with folate (**2**) and vitamin B_{12} (**4**), it has recently been shown that biotin receptors are overexpressed in many cancer cells.[75] The research group of Ojima described several conjugates of biotin (**3**) to fluorescein, coumarin, a taxoid-fluorescein derivative, and the improved taxoid SB-T-1214.[51] The authors were able to show that L1210FR leukemia cells could take up biotin–fluorescein conjugates efficiently. 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 cleaved efficiently, thereby resulting in the green fluorescence labeling of microtubule bundles. Finally, whilst the free taxoid exhibited low nanomolar cytotoxicity ($IC_{50} = 9.5$–10.7 nM) against biotin receptor positive and negative cell lines, the targeted cytotoxic selectively killed biotin receptor positive cells with a potency equal to that of the free taxoid ($IC_{50} = 8.8$ nM).

2.3. Substrate Analogues

Prostate-specific membrane antigen (PSMA, also known as glutamate carboxypeptidase II) is a well-established marker of prostate cancer and also occurs in the neovasculature of many solid tumors.[121,122] 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.[123] Analogues of *N*-acetylaspartyl glutamate (i.e. the natural substrate of PSMA, Scheme 4) **8**–**11** are nanomolar inhibitors of the enzyme, and have been used as homing ligands for tumor imaging,[124,125] targeted delivery of cytotoxics,[52,53,126,127] and also hapten-based immunotherapy.[128]

The first PSMA ligand–drug conjugate consisting of a urea-based PSMA inhibitor, a glutaryl linker, and doxorubicin was described by Kozikowski and co-workers.[53] The product efficiently blocked the binding of a radioactive competitor to recombinant PSMA dimer. In vitro cytotoxicity, however, was markedly lower compared to that of free



Scheme 4. a) PSMA-catalyzed cleavage of *N*-acetylaspartyl glutamate (NAAG) to *N*-acetylaspartate and glutamate.[124] b) NAAG-analogue inhibitors of PSMA **8**–**11** capable of delivering payloads into solid tumors.[52,53,124–128] Groups which tolerate the attachment of a payload are marked in blue. c) A conjugate of the PSMA-inhibitor DUPA (**12**) with tubulysin B connected through a peptide spacer and self-immolative disulfide linker.[52,126]

doxorubicin (at 5 μM, only 30% of C4-2 cell proliferation was inhibited), possibly because of the stable nature of the amide linker.

Very potent PSMA-based targeted cytotoxics have since been reported by the research group of Low.[52,126] By using 2-[3-(1,3-dicarboxypropyl)ureido]pentanedioic acid (DUPA, **11**; $K_i = 8$ nM against the purified enzyme[52]) 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.[52] DUPA–^{99m}Tc was investigated as a radio-imaging agent ($K_D = 14$ nM towards LNCaP cells in vitro).[52] Besides high tumor uptake (11.2% ID g^{-1}) strong accumulation was also observed in the kidneys (28.9% ID g^{-1}), which, however, can at least partly be explained by the expression of PSMA in murine kidneys. Finally, a conjugate of tubulysin B connected to DUPA (**11**) through a self-immolative disulfide linker **12** showed potent inhibition of [^{3}H]-thymidine incorporation ($IC_{50} = 3$ nM) in LNCaP cells in vitro and sustained tumor regression in LNCaP xenografts with no observable toxicity in vivo.[52] DUPA–drug conjugates with desacetylvinblastine ($IC_{50} = 31$ nM), camptotecin ($IC_{50} = 115$ nM), verrucarin A ($IC_{50} > 1$ μM), tubulysins H I, II, and III ($IC_{50} = 5$–24 nM), and didemnin B ($IC_{50} = 107$ nM) have been investigated, and some exhibited potent activity against LNCaP cells.[126] An approach related to targeted delivery described by Schultz and co-workers merits a mention 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.[128]

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.[129] In the case of tumor-targeting applications, one would typically raise antibodies to the extracellular portion of a membrane protein, which is overexpressed at the tumor site relative to normal tissue. More recently, components of the modified extracellular matrix at the site of disease,[130,131] as well as markers of angiogenesis located at newly formed blood vessels[132,133] 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[30] and by the difficulty of reaching cancer cells which are far away from the nearest tumor blood vessels.[34] By contrast, markers expressed in the tumor neovasculature and in the subendothelial extracellular matrix may be more accessible, abundant, and genetically stable, thus allowing an efficient tumor targeting with long residence time of the ligand at the site of disease.[18]

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 also overexpressed in the majority of kidney cancer cells as a result of von Hippel–Lindau mutations),[134,135] placental alkaline phosphatase (an excellent marker of ovarian cancer),[136] matrix metalloproteinase-3,[137,138] and PSMA in prostate cancer,[121,122] to name just a few.

Validated receptor tyrosine kinases, such as EGFR and HER2/neu,[139] 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.[140] 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 the design and screening of chemical libraries.[141]

It is worth mentioning that, in principle, the selective expression of certain enzymatic activities at the site of the 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 recently.[142]

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 discovery of small molecule ligands often relies on high-throughput screening procedures with large compound collections (up to 1×10^6 molecules), followed by extensive medicinal chemistry optimization. This approach is not only extremely demanding in terms of time, management, logistics, and costs, but often fails to yield hits with sufficient affinity, especially to "difficult" targets (such as those involved in protein–protein interactions).[143,144] Alternatively, approaches based on computational design or virtual screening are also widely practiced.[145]

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"). After incubation of the library with immobilized antigen ("panning"), nonbinders are washed off and specific binders recovered by elution. The attached genetic information is amplified by the polymerase chain reaction (PCR) and then sequenced to establish the identity of the binder.[16] The power of such approaches is illustrated by the widespread use of phage display and similar display approaches.[146,147]

In the following we will discuss methods based on the principle of a genotype–phenotype linkage, which can be used for the identification of low-molecular-weight binders, more specifically short peptides and nonpeptidic small molecules. Since DNA has emerged as the carrier of information regarding the ligand identity in all of these settings, 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. Firstly, they do not require the costly infrastructure associated with high-throughput screening, thus also allowing 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 attachment of the payload.

Some DNA-encoded library technologies have already yielded binders ($M = 500$–$2000\ \mathrm{g\,mol^{-1}}$) capable of selective accumulation in tumors in vivo. Others are very promising in our view, but a definite proof has not yet been established.

4.1. DNA-Encoded Libraries of Peptides

Phage display is widely used for the construction of linear short-peptide libraries. In brief, filamentous phage (e.g. M13) can be engineered to express foreign amino acid sequences fused to certain coat proteins without compromising the phage infectivity and propagation ability, thus establishing a stable genotype–phenotype linkage.[146,148] This approach has been used in the identification of linear peptide ligands for various tumor markers and has been covered in recent reviews.[149,150] Although often successful in vitro, only a few peptides showed adequate targeting performance in tumor models in vivo. When biodistributions of labeled peptides were performed, tumor uptake was often shown to be very low, with high accumulation in the liver[151] or kidneys.[149,152]

Nevertheless, some peptides derived from phage display have been used for the construction of drug conjugates and tested in vivo. A conjugate of the integrin binding peptide RGD-4C with doxorubicin exhibited reduced toxicity and more potent retardation of tumor growth than doxorubicin alone in a murine model of cancer.[153] A conjugate of an EphA2 binding peptide ($K_D = 200$ nM) and paclitaxel connected through an ester linkage has recently been shown to induce a more potent antitumor effect than paclitaxel alone in mice.[154]

Other peptides have shown some success as imaging agents, thus suggesting potential applications as homing ligands in drug conjugates. The RMS-II peptide, for example, labeled rhabdomyosarcoma lymphatics and blood vessels in vivo.[155] Similarly, a peptide raised against the prostate cancer marker hepsin[156] allowed near-infrared fluorescence mediated tomography (FMT) imaging of LNCaP xenografts in mice. The ^{64}Cu-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) derivative of AE105 exhibited good (8.1 % ID g^{-1}) accumulation in U87MG glioblatoma tumors in rodents.[157,158]

High kidney and liver uptake can compromise the in vivo performance of peptide-based delivery approaches. Nonetheless, experience gained from somatostatin analogues and other peptide-based pharmacodelivery strategies suggest that an efficient and selective tumor uptake is possible when abundant expression of an accessible target and high binding affinity occur.[56] 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,[159–161] for example, has successfully been used for the identification of cyclic RGD ligands for different integrins[162] and a tumor-homing peptide that targets lymphatic vessels.[163] Winter, Heinis, and co-workers have developed an approach by which linear peptide precursors containing three Cys residues were cyclized with a trivalent reactive chemical scaffold (Figure 4a).[164] A potent and highly specific inhibitor of human plasma kallikrein (**13**, Scheme 5; $K_i = 1.5$ nM),[164] and an inhibitor of the urokinase-type plasminogen activator

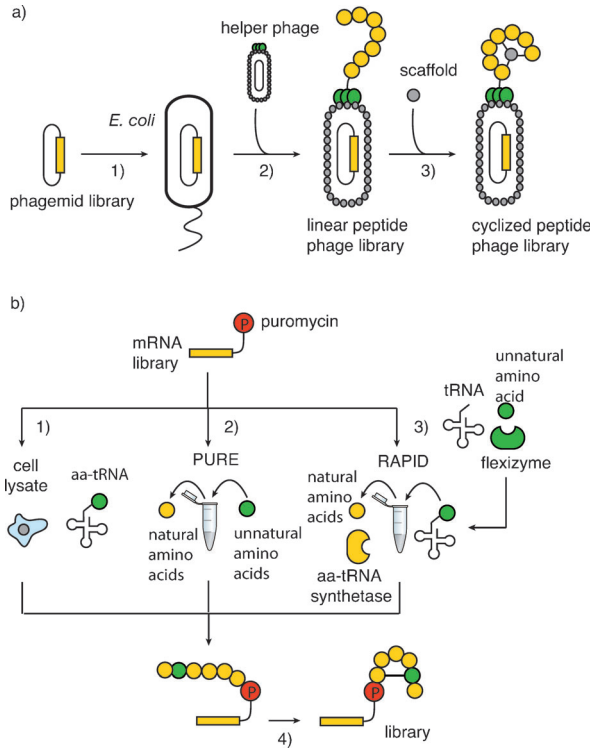
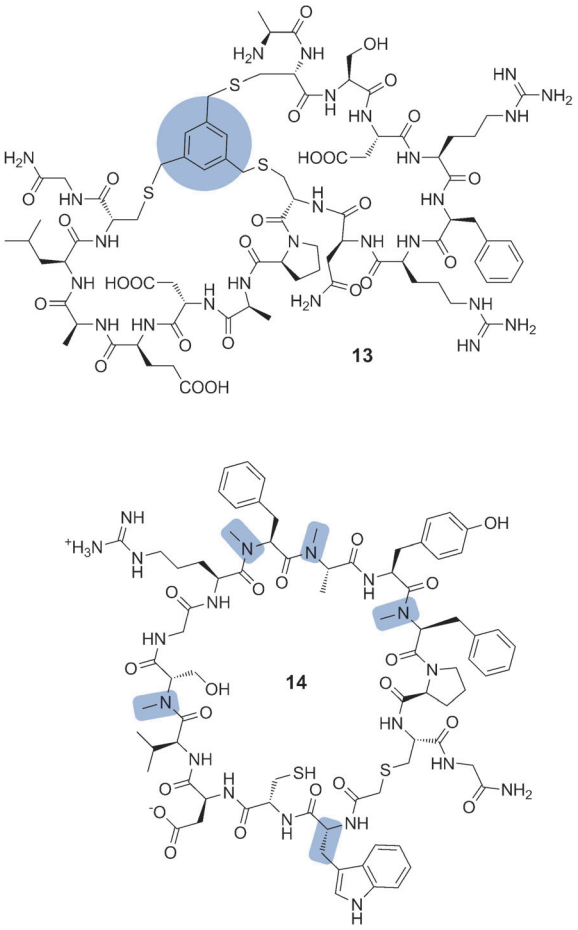


Figure 4. a) Construction of phage libraries of linear or circular peptides in bacteria.[146,148,164] 1) Phagemid libraries are assembled by PCR and transfected into *E. coli*. 2) Superinfection with a helper phage facilitates expression of phage-bound peptides. 3) Cyclization of the phage-bound peptides with reactive chemical scaffolds yields the library. b) Synthesis of peptide libraries containing unnatural amino acids by using mRNA display and in vitro translation.[167] 1) Using whole cell lysate and nonsense codon suppressing aminoacyl-tRNA (aa-tRNA).[168,169] 2) Using PURE.[170,171] Unnatural amino acids (green circles) can be incorporated by withdrawing natural amino acids (yellow circles) and adding unnatural ones, which can be loaded onto tRNA by natural aa-tRNA synthetases. 3) Using the RAPID system.[172–176] Selected natural amino acids (yellow circles) and aa-tRNA synthetases (yellow oval shape) are removed from the PURE system, and tRNA loaded with unnatural amino acids (green circles) is added. The synthesis of unnatural amino acid containing aa-tRNA is conveniently performed using flexizymes (green oval shape). 4) Cyclization of the peptide, e.g., through the side chains of unnatural amino acids.



Scheme 5. Examples of molecules isolated from DNA-encoded peptide libraries. Human plasma kallikrein inhibitor **13**[164] and Akt2 inhibitor **14**.[175] Unnatural structural features are highlighted in blue.

(uPA), a serine protease that is implicated in tumor growth and invasion ($K_i = 53$ nM), were identified by using this method.[165] More recently, an improved inhibitor of kallikrein ($K_i = 0.3$ nM) was reported.[166]

Several methods relying on in vitro translation systems for the construction of DNA-encoded cyclic peptide libraries have also been reported (Figure 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.[167]

Rabbit reticulocyte lysate has been shown to efficiently translate a library of approximately 1.2×10^{11} peptides linked to their respective encoding mRNAs. Cyclization was achieved through Cys residues encoded in the sequence. Potent α-thrombin binders with (K_D values down to 166 nM) could be isolated by using this approach.[168] It is further possible to include unnatural amino acids into similar systems by suppression of the nonsense codon.[169] A very strong cyclic inhibitor of Gαi1 ($K_i = 2.1$ nM) was identified by using such an approach. A more extensive reprogramming of the genetic code has to be contrived to construct libraries capable of simultaneously containing several different unnatural amino acids. Szostak and co-workers have thus used an in vitro translation system reconstituted from purified components (PURE)[170] 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.[171] The most advanced system for the construction of DNA-encoded cyclic peptide libraries containing numerous unnatural amino acids to date is the random nonstandard 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 aminoacyl-tRNA synthesis is achieved through a flexible ribozyme (flexizyme),[172,173] which can add almost arbitrary amino acids onto tRNAs.[174] The use of libraries based on thioether cyclization of the backbone enabled potent (IC_{50} values in the range of 100 nM)

and isoform-selective inhibitors of Akt2 (e.g. **14**, Scheme 5)[175] as well as a potent binder of ubiquitin ligase E6AP ($K_D = 0.60$ nM)[176] to be identified.

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 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 that synthetic peptides could be encoded with oligonucleotides (merely acting as amplifiable "bar codes", rather than as genes for biosynthesis) on the same solid support.[177] Although this article marked the birth of DNA-encoded library synthesis, it was only a decade later that this concept was implemented in 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 practiced widely (Figure 5a).[178–183] In brief, this approach features the following sequential steps:[184]

a) A first set of chemical compounds is coupled to short DNA fragments coding for the respective building block.
b) The products of the first step are pooled and split into separate reaction vessels.
c) 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).
d) Pooling of the last encoding step yields the final DNA-encoded chemical library.

Steps (b) and (c) can in principle be repeated several times to generate libraries with multiple building blocks and between 10^5 and 10^9 encoded small molecules. In practice, however, multiple split and pool cycles are unavoidably associated with decreasing library purities, as unwanted side products accumulate. Also, despite the tremendous progress made in the field of high-throughput DNA sequencing,

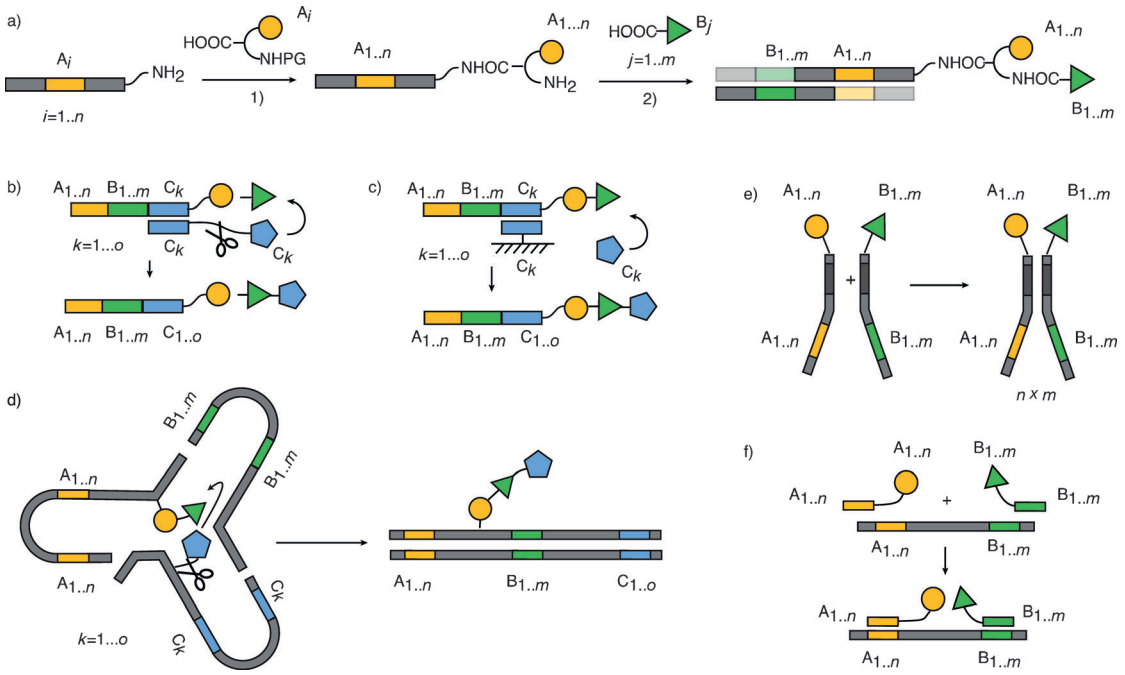


Figure 5. a) Generation of a DNA-encoded library by sequential split and pool synthesis using peptide chemistry. 1) Coupling of building blocks A_i to oligonucleotides coding for A_i. Deprotection, purification reactions, pool, and split into m aliquots. 2) For each aliquot, coupling with one building block B_j. Precipitation of DNA and encoding reaction step by Klenow polymerization. Pooling of all the reactions to obtain the final library. b) DNA-templated library synthesis (DTS) as used by Liu and co-workers.[186–189] DNA-bound building blocks (e.g. C_k) anneal with complementary sequences on the coding DNA strand. The spacial proximity promotes the reaction of C_k with the previous building blocks. Cleavage of the linker between the building blocks and their coding DNA followed by a workup step finishes the cycle. c) Construction of a library by DNA routing.[190,191] Subsets of the library are sequentially immobilized on beads bearing sequences complementary to the coding sequences. Each immobilized sublibrary is coupled with the corresponding building block, eluted, and mixed with the rest of the library. For this technique, oligonucleotides complementary to all the coding sequences of the library need to be available on the solid support. d) Construction of a library by DTS in a "yoctoliter reactor".[192] Here, three-way hairpin junctions promote reaction through spatial proximity. e) Encoded self-assembling chemical (ESAC) libraries.[193–197] Two sublibraries bearing different building blocks are hybridized through a constant domain, which allows the construction of high-quality combinatorial libraries with a size of $n \times m$ 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.[198,199]

sampling libraries containing dozens of millions of compounds remains an expensive task.

The synthesis of DNA-encoded chemical libraries relies on the availablility of highly efficient coupling reactions, which are compatible with the structure of DNA and can proceed in the presence of water. Diels–Alder, 1,3-dipolar Cu^{I}-catalyzed azide–alkyne cycloaddition, nucleophilic aromatic substitution, reductive amination, and peptide coupling reactions have been used to this end.[182,183,185]

An alternative to the manual split and pool assembly of DNA-encoded chemical libraries is 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 proximity through formation of a double strand (Figure 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.[186–189]

The research group of Harbury described a strategy termed "DNA routing" (Figure 5c).[190,191] 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 co-workers used three-way DNA hairpin junctions to transfer appropriate donor chemical moieties to an acceptor (Figure 5d).[192]

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, library formats presenting two separate molecular entities ("dual pharmacophore libraries") have also been developed. Our research group described the construction of encoded self-assembling chemical libraries (ESAC) that allowed the combinatorial assembly of encoded sublibraries through formation of DNA heteroduplexes (Figure 5e).[193–197] 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 that facilitates duplex formation between the sublibraries and a variable sequence that represents the individual chemical moieties. Mixing equimolar amounts of every library member results in the spontaneous assembly of a combinatorial library containing every possible combination of the building blocks. Since only a single synthetic step is required for making the sublibraries, 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 be directly used in PCR amplification after selections (Figure 5f).[198,199] In both approaches, the flexibility of the linkers between the DNA and the displayed molecules allows the simultaneous exploration of two different binding pockets on the same target protein, thereby yielding a significantly increased binding affinity because of the chelate effect.[197] 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").[181]

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 by using this approach.[184] Our research 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.[200] A CAIX ligand isolated from a $1x10^6$ member DNA-encoded chemical library **15** (Figure 6a; inhibits hydrolysis of *p*-nitrophenyl acetate by CAIX with $IC_{50} = 260$ nM) selectively accumulated in the tumor upon injection in a mouse model of human cancer (Figure 6b).[185] We and others[201,202] are currently working on improved CAIX-targeting agents, for the delivery of potent cytotoxic molecules to renal-cell carcinoma.

5. Cytotoxic Payloads and Linkers

Cytotoxic payloads for targeted-delivery applications (Table 1)[203–207] 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,[208] vinca-alkaloids,[90] taxoids,[114,209] and anthracyclines.[61–63] Despite their relatively low toxicity, early attempts often relied on doxorubicin and its derivatives as well as methotrexate, as their chemical structures permitted facile modification and the drugs were available in large quantities.[53,61–63,153] 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 analogues, such as the auristatins,[210,211] and cemadotin,[25,212] maytansinoids,[213,214] calicheamicins,[204,215] pyrrolobenzodiazepines,[216,217] duocarmycins,[206,218] and tubulysins.[52,92,93,207,219]

The cytotoxic drug should remain stably attached to the targeting ligand whilst the conjugate is in the circulation, but be released efficiently when the construct reaches the intended site of action. Linkages undergoing pH-depen-

Table 1: Growth inhibition data of cytotoxic agents representing the most common payload families. The IC_{50} values for the most sensitive cell lines (minimum), least sensitive cell lines (maximum), and the median IC_{50} values are given.

Compound	Family	IC_{50} [M]			Cell lines tested	Reference
		minimum	maximum	median		
methotrexate[a]	antifolates	2.4×10^{-8}	2.5×10^{-4}	7.3×10^{-8}	97	[203]
doxorubicin[a]	anthracyclines	2.5×10^{-9}	1.3×10^{-5}	8.4×10^{-8}	85	[203]
paclitaxel[a]	taxoids	2.8×10^{-9}	1.6×10^{-6}	9.6×10^{-9}	94	[203]
vinblastine[a]	vinca-alkaloids	2.5×10^{-10}	2.7×10^{-6}	6.4×10^{-10}	99	[203]
maytansine[a]	maytansinoids	1.2×10^{-9}	2.0×10^{-5}	3.3×10^{-9}	74	[203]
dolastatin[a]	dolastatins	3.2×10^{-11}	6.8×10^{-5}	7.1×10^{-10}	76	[203]
auristatin PE[a]	dolastatins	8.7×10^{-11}	1.0×10^{-5}	4.1×10^{-10}	60	[203]
–	calicheamicins	$<5.0\times10^{-11}$ in HeLa cells			1	[204]
SJG-136	pyrrolobenzo-diazepines	2.3×10^{-11}	5.0×10^{-6}	4.7×10^{-9}	8	[205]
duocarmycin SA[b]	duocarmycins	9.3×10^{-13}	5.7×10^{-9}	6.2×10^{-10}	15	[206]
tubulysin D	tubulysins	3.1×10^{-12}	6.7×10^{-10}	8.9×10^{-12}	4	[207]

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

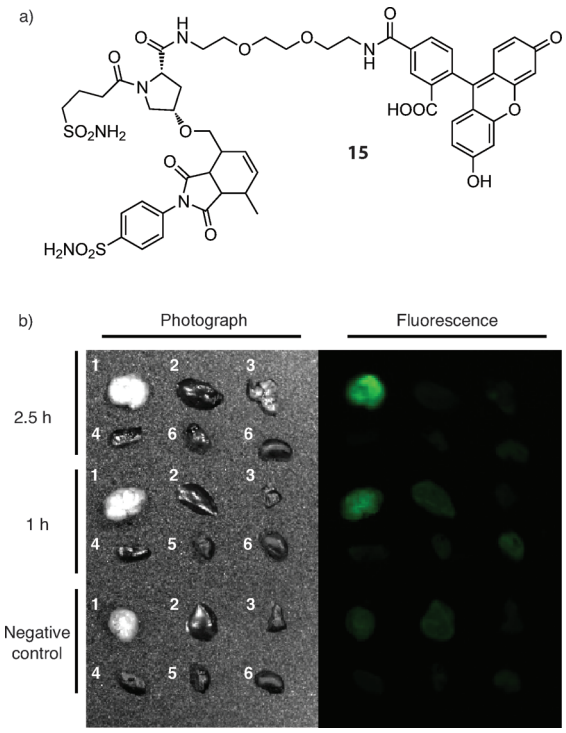
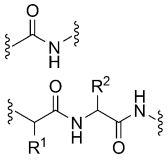
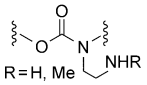
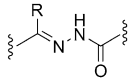
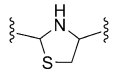
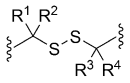
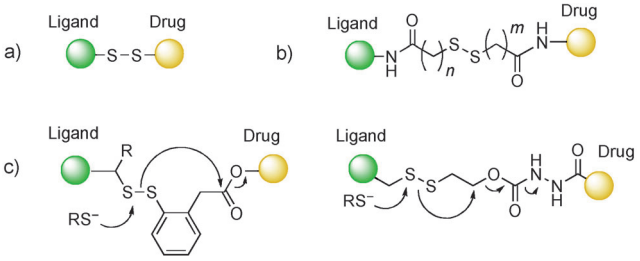


Figure 6. a) The fluorescein conjugate of a CAIX ligand **15**, which was isolated from a 1x10^6 member DNA-encoded chemical library, accumulates in solid tumors in mice after intravenous injection. b) Photographs and fluorescence pictures of extracted organs. 1) tumor, 2) liver, 3) lung, 4) spleen, 5) heart, 6) kidney. Control = *N*-(2-(2-(2-aminoethoxy)ethoxy)ethyl)carboxyfluoresceinamide.[185]

Table 2: Commonly used cleavable linkages for the construction of ligand-targeted cytotoxics.

Linkage	Structure	Release mechanism
amide		hydrolysis
ester		hydrolysis
carbamate		hydrolysis
hydrazone		hydrolysis
thiazolidine		hydrolysis
disulfide		reduction



Scheme 6. a) Traceless disulfide linkage.[25] b) Disulfide linker connecting ligand and payload through amide bonds.[223] c) Self-immolative linkers based on disulfides as the cleavable linkage.[126,226]

dent,[90,212,215] reductive,[25,77,220] and enzyme-catalyzed cleavage[221,222] have been developed towards this aim (Table 2). When suitable functional groups are present directly on the ligand and payload (e.g. thiols), a "linkerless" connection can be established, thus facilitating "traceless" release of the drug (Scheme 6a).[25,212] When this is not possible, cleavable linkers are required as adaptors to connect the ligand to the payload (Scheme 6b). In this case, ligands or drugs may be released with covalent modifications,[223] which can potentially complicate pharmacokinetics or give rise to immunogenicity. Most linkage techniques 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.

Initial attempts to produce targeted drug conjugates often relied on dicarboxylic acid spacers coupled to the drug and the targeting vehicle as esters or amides.[61–63] If such constructs reach the lysosome, cleavage of the amide or ester bond by lysosomal proteases or esterases can be envisioned. Also carbamate based linkers have been described.[73] 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,[53,73,74,224] thus suggesting inefficient cleavage. Interestingly, antibody–drug conjugates 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.[220] 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 being widely employed.[221,222]

Hydrazones are acid labile and hydrolyzed in the lysosome and certain endosomes.[90,215] Our research group has recently proposed the use of thiazolidines as hydrolytically labile traceless linkages. 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.[212]

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.[77] In their simplest form, they can be established between thiol groups on the drug (e.g. cemadotin-SH) and the targeting vehicle,[25] thereby facilitating a traceless separation between the carrier and payload (Scheme 6a). However, alkyl spacers with disulfide groups are often used (Scheme 6b).[14,15,225] Their release kinetics can be modulated by the steric bulk around the disulfide bond.[220] Self-immolative linker systems based on 2-mercaptoethyl carbamates[126] or 2-mercaptophenylacetate[226] can be used when the cytotoxic payload does not tolerate the attachment of an adaptor without losing activity (Scheme 6c). Traceless drug linkage technologies may be preferable, though, as the elimination of self-immolative linkers is not an instantaneous process.

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.[77,113] Similarly, HER2 internalization does not facilitate the efficient cleavage of disulfide linkers, at least in certain cell lines.[227] 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. It is still difficult with current methods to isolate specific ligands to some of the most attractive accessible markers of pathology (e.g. components of the modified extracellular matrix). It has been shown in the case of antibody phage display that the use of library subsets with only millions of antibodies does not typically yield high affinity binders (or yield binding specificities at all).[228] Libraries of billions of antibodies, on the other hand, 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.

Direct comparison of antibody-based targeting of disease with the corresponding pretargeting strategies reveal a striking benefit associated with small organic ligands, in terms of rapid tissue distribution, efficient uptake at the site of disease, and rapid clearance. While important for mechanistic studies, pretargeting technologies are difficult to implement in industrial and clinical settings, because of their multicomponent nature and 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 need, at some stage, to be removed from the circulation. Indeed, in the absence of metabolic degradation, virtually 100% of injected drugs end up, at some time point, in clearance organs (most importantly, the liver and 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 the 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.

Intensive research is currently exploring various classes of cytotoxic drugs and linker strategies. Many potent drugs target tubulin (thus, having an impact on cell division),[229] but these agents may be less effective against dormant tumor cells. For this reason, specific 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-grove 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.[230–232]

In cancer chemotherapy, cytotoxic drugs are commonly believed to mainly affect tumor cells. However, in principle, profound anticancer 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,[233] 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,[234] 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 methods (e.g. positron emission tomography) will be increasingly important to evaluate the targeting performance, thereby 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 anticancer research, as many of the targets, linkers, and drugs which are currently used with antibody vehicles should be readily adaptable to work in the context of fully synthetic, next-generation targeted cytotoxics.

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