REVIEWS

Antibody−Drug Conjugates: Linking Cytotoxic Payloads to Monoclonal Antibodies

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Antibody−drug conjugates (ADCs) combine the specificity of monoclonal antibodies (mAbs) with the potency of cytotoxic molecules, thereby taking advantage of the best characteristics of both components. Along with the development of the mAbs and cytotoxins, the design of chemical linkers to covalently bind these building blocks is making rapid progress but remains challenging. Recent advances have resulted in linkers having increased stability in the bloodstream while allowing efficient payload release within the tumor cell.

INTRODUCTION

After cardiovascular diseases, cancer represents the second most common cause of death in the Western world and is a major area of focus for the pharmaceutical industry. Surgery and radiotherapy are generally used when a tumor is localized to a certain tissue, but chemotherapy is needed when metastasis has occurred. Despite extensive research, most anticancer drugs have nonspecific toxicity. By targeting the cell cycle and thereby killing rapidly proliferating cells, they do not explicitly discriminate between healthy and tumor tissues and only gain a limited selectivity for malignant cells. Such cytotoxic drugs have a narrow therapeutic window, which limits their efficacy and results in severe side effects. Due to a lack of selectivity, drug concentrations that would eradicate the tumor can often not be used. In addition, tumors can develop resistance against anticancer drugs after prolonged treatment. Therefore, achieving improved tumor selectivity through targeting of cytotoxic drugs to the cancer cells is needed.

A promising approach to achieving a more selective treatment is targeted prodrug therapy (*1*). Antibody−drug conjugates (ADCs) are ideal candidates for such prodrugs. ADCs are monoclonal antibodies (mAbs) linked to cell-killing drugs. Thanks to their high binding specificity for tumor-specific antigens, mAbs can be used as vehicles to target cell-killing payloads to tumor cells. Unique or overexpressed, tumor-specific antigens can be found in a wide range of human tumor cells (*2*). Some mAbs have the ability to recognize and specifically bind to these tumor-associated antigens. They can be used as single agents for the treatment of cancer through binding to cancer-cell-specific antigens and induction of an immunological response against the target cancer cell (*3*). However, therapeutic efficacy is often limited by the extent to which the antibody leads to cell death. Monoclonal antibodies are extremely discriminating for their targets but sometimes therapeutically ineffective on their own. The insufficient efficiency of most naked mAbs in cancer therapy has been circumvented by arming the immunoglobulin with radioactive isotopes (*4*) or cytotoxic drugs (*5*−*7*) (Figure 1), yielding highly specific ADCs.

ADCs can be viewed as sophisticated delivery systems for antitumor cytotoxic drugs. The mAb guides the toxin precursor to the target cancer cell, where the prodrug can be converted chemically and/or enzymatically to the parent drug and unfold its cytotoxic activity. The ADC is exposed to different conditions on its journey from the blood vessels to the molecular target in the tumor tissue. The mode of action at cellular or molecular level is complex, with each step bringing its own challenges:

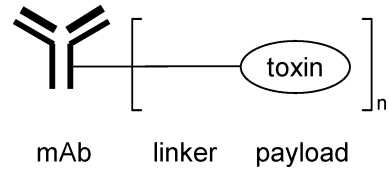

Figure 1. Schematic representation of an antibody−drug conjugate.

a. Circulation. The ADC must behave like a naked antibody when circulating in the plasma. In particular, the linker must be stable in the bloodstream to limit the damage to healthy tissue. Decomposition or decay would release the cytotoxin before being delivered to the target site.

b. Antigen Binding. It is necessary that the conjugated mAb retains high immunoaffinity. Attaching the cytotoxic compound to the mAb must thus not disturb its binding specificity.

c. Internalization. A sufficient intracellular concentration of the drug must be achieved. This is challenging because antigen targets on cell surfaces are often present in limited numbers and the internalization process for antigen−antibody complexes is frequently inefficient.

d. Drug Release. Once internalized, the ADC has to efficiently release the original cytotoxic drug in its active form inside the tumor cell.

e. Drug Action. The inherent potency of the released drug must be sufficient to kill the tumor cell, even at low concentration. To achieve significant cytotoxicity, use of very potent drugs with subnanomolar IC_{50} (as free drug) becomes necessary. It has been shown that compounds found to be too toxic when tested as a stand-alone chemotherapy agent are suitable candidates for ADC payloads. These toxins can be 100 to 1000 times more cytotoxic than traditional anticancer agents.

The molar loading of the drug on the mAb can be tuned to achieve the desired potency. Because of points (c) and (d), linkage of several drugs is generally necessary to achieve significant cytotoxicity. However, if too many cytotoxic molecules are attached

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Chart 1

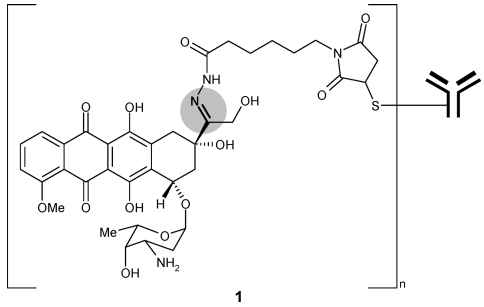
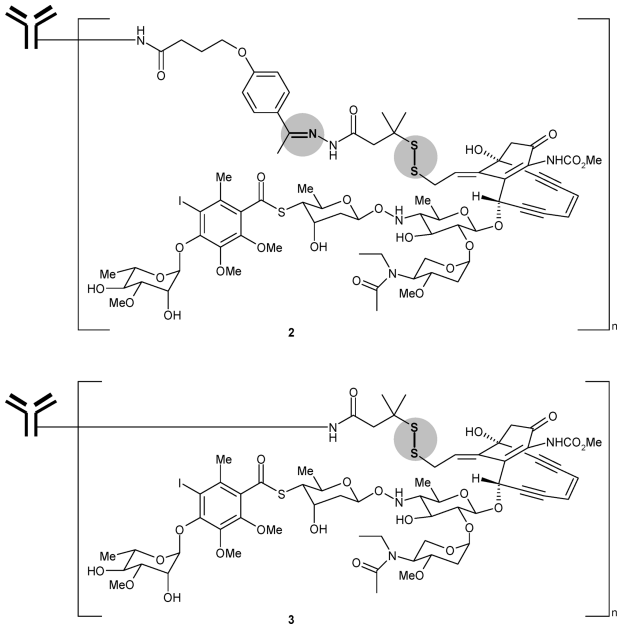


Chart 2



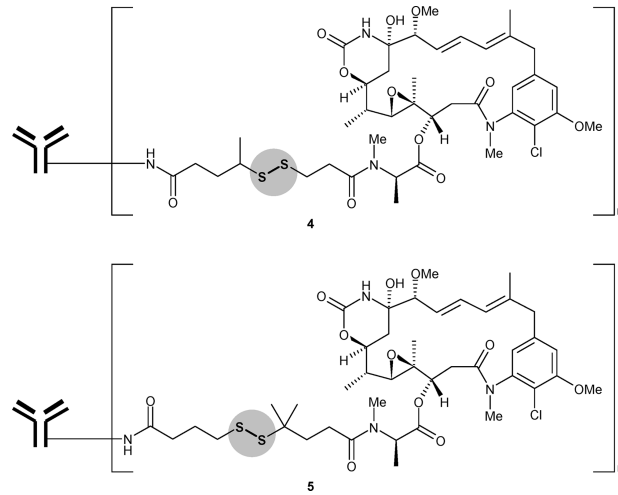
to the antibody, the body may recognize the conjugate as a damaged form of the protein and quickly clears it from the system, proving points (a) and/or (b) to be problematic. The extent of drug substitution may also affect pharmacokinetics, so that most of the time 2−4 drugs per antibody give the best therapeutic window (*8, 9*). To avoid the interference with the antigen recognition, the drugs are ideally linked at the Fc or constant region of the mAb that does not participate in binding to the antigen (point (b)). To regain the full cytotoxic potential of the drugs, cleavable linkers releasing the drug molecule at the target site in unmodified form were developed to address point (d), although these also affect the stability of the construct. Therefore, the right balance between plasma stability and efficient drug release at the target cell has to be found.

The insights gained during ADC development within the past decade have resulted in a number of new strategies and drugs (*4*−*7*). This review focuses on linker technologies used to attach low molecular weight cytotoxic drugs to mAbs. The linker is a small but central part of ADCs and as such accounts for stability in circulation, favorable pharmacokinetic properties, and efficient release of toxic agent at the tumor site.

CHEMICALLY LABILE LINKERS

Selective linker cleavage and payload release is based upon the differential properties between the plasma and some cytoplasmic compartments. Linkers were chosen that were stable in the blood's neutral pH environment but were getting cleaved

Chart 3



once the ADC entered the lower pH environment found inside a cell. The focus in the early days of ADC development was on acid-cleavable hydrazone linkers that were relatively stable at neutral pH (bloodstream pH 7.3−7.5), but were undergoing hydrolysis once internalized into the mildly acidic endosomes (pH 5.0−6.5) and lysosomes (pH 4.5−5.0).

In the 1990s, Bristol-Myers Squibb scientists covalently linked doxorubicin, an intercalating agent that blocks DNA replication and is used as chemotherapeutic agent, to the humanized mAb BR96 (*10*). Doxorubicin was connected over a (6-maleimidocaproyl)hydrazone linker to cysteine residues of the Lewis-Y specific mAb. The preclinical activities of BR96-doxorubicin (**1**, Chart 1) were remarkable, but despite the use of eight drugs per mAb molecule, the amount of conjugate needed to achieve these effects *in vivo* were high (>100 mg/kg). This was presumably due to the relatively low potency of doxorubicin (IC_{50} of 0.1−0.2 μM for human carcinoma lines (*11*), whereas subnanomolar activities are now typically seen for ADC payloads). Moreover, the half-life of the drug in the blood was approximately 43 h, which is short compared to the half-lives of several days to weeks of the naked BR96 mAb in humans. The compound later failed to show sufficient clinical efficacy.

The first and so far sole mAb linked to a cytotoxic payload that has been given regulatory approval is Wyeth's gemtuzumab ozogamicin (**2**, Mylotarg; Chart 2) (*12*−*14*). The drug was approved by the US Food and Drug Administration (FDA) in 2000 for use in patients over 60 suffering from relapsed acute myelocytic leukemia (AML), the most common form of leukemia in adults. Gemtuzumab ozogamicin (**2**) consists of *N*-acetyl-γ-calicheamicin (an enediyne antibiotic that binds to the minor groove of DNA and causes double-strand breaks leading to apoptosis) covalently attached to humanized anti-CD33 IgG4 κ antibody (hP67.6) via a bifunctional linker. The 4-(4-acetylphenoxy)butanoic acid moiety allows for attachment to surface-exposed lysines of the antibody over an amide bond and forms an acyl hydrazone linkage with *N*-acetyl-γ-calicheamicin dimethyl hydrazide. Upon internalization of the ADC, the calicheamicin prodrug is released by hydrolysis of the hydrazone in lysosomes of the CD33-positive target cells. The hydrolyzability of an unconjugated intermediate at 37 °C over 24 h increased from 6% at pH 7.4 to 97% at pH 4.5 (*15*). The enediyne drug then gets activated by reductive cleavage of the disulfide bond. This disulfide linkage has been stabilized by two methyl groups to prevent premature release of calicheamicin by circulating reduced thiols, such as glutathione. In most preclinical models, the hydrazone linkage produced ADCs with higher potency than the corresponding amide-bearing conjugate **3**, providing evidence that with this mAb the disulfide alone is insufficient for

Scheme 1. Presumed Release Mechanism of MMAE (7) from ADC 6 Incorporating a Valine-Citrulline Peptidic Linker

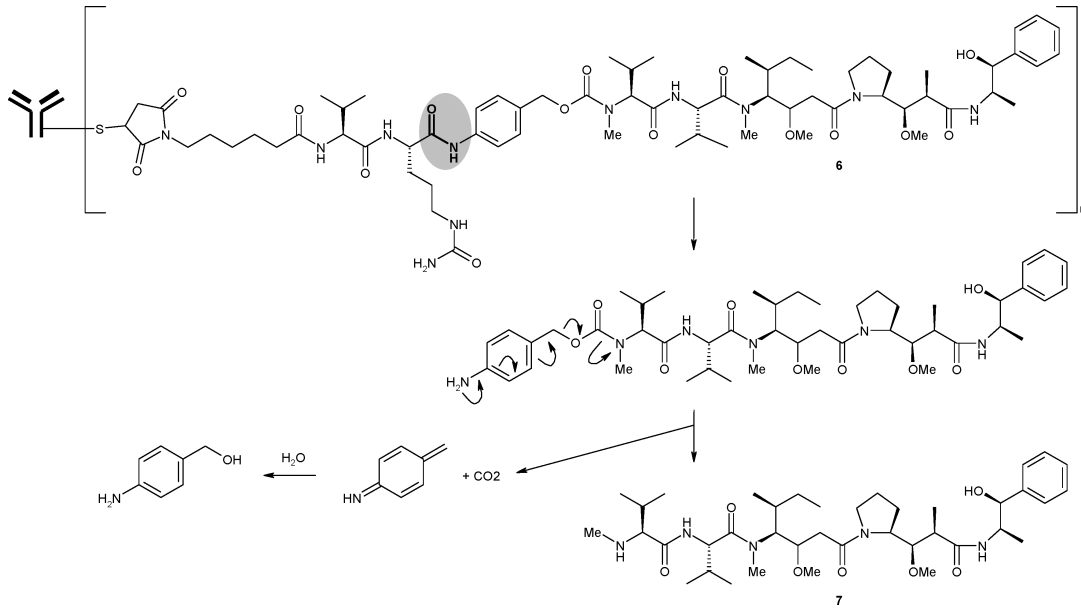
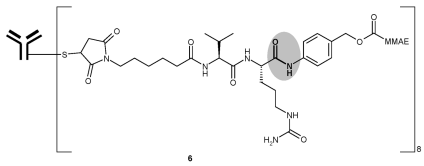
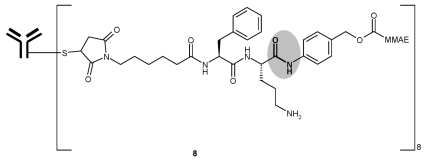
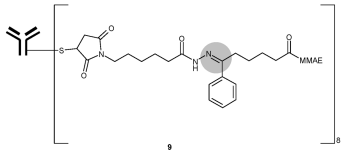


Table 1. Compared Stabilities of Enzyme-Labile and Chemically Labile Linkers in ADCs with MMAE Conjugated to cBR96 mAb

ADC	Linker	Half-live mouse plasma	Half-live human plasma	Maximum tolerated dose
	Valine-citrulline	30 days*	230 days*	30 mg/kg
	Phenylalanine-lysine	12.5 days*	80 days*	n/a
	Hydrazone	2.1 days	2.6 days	15 mg/kg

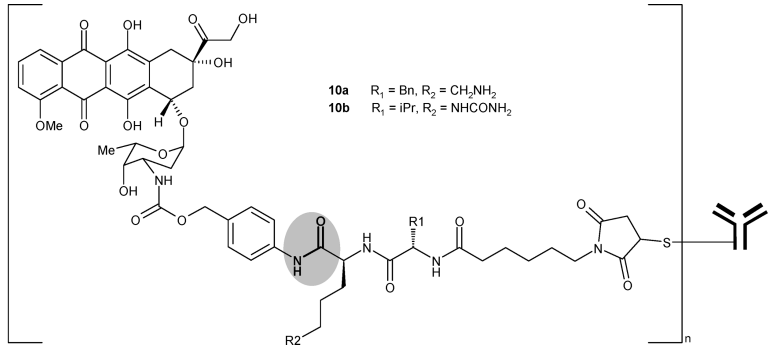
* projected half-life

efficient release of the drug in the target cell. Interestingly, with the murine mAb CTM01, ADC **3** showed activities equal to or even greater than that of the corresponding hydrazone conjugate **2** in several *in vitro* and *in vivo* tumor models, revealing that this conclusion is not general (*16*). In clinical trials, however, compound **3** had only limited evidence of activity.

Mylotarg has met limited successes due to a narrow therapeutic window. The linker technology based on pH-dependent release mechanism is possibly not sufficiently stable and too much of the drug is being released in the bloodstream, as pharmacokinetic data have shown that the mean half-life of Mylotarg is 72 h (*15*). Nonetheless, the development of CMC-544 (inotuzumab ozogamicin), which is a humanized anti-CD22 mAb identically attached to *N*-acetyl-γ-calicheamicin dimethyl hydrazide via the acid-labile 4-(4′-acetylphenoxy)butanoic acid linker is ongoing at Wyeth. Although this ADC is closely related to Mylotarg, good stability was shown in both human plasma and serum (rate of hydrolysis of 1.5−2%/day over 4 days) (*17, 18*).

Disulfide bonds can be an alternative to acid-labile hydrazone linkers. In this case, the release is attributed to the high intracellular concentration of glutathione. This thiol-containing tripeptide is present in micromolar concentrations in the blood, whereas its concentration in the cytoplasma is in the millimolar range (up to 1000-fold higher). This is especially true for tumor cells, where irregular blood flow leads to hypoxic state (decreased oxygen level), resulting in enhanced activity of reductive enzymes and therefore even higher glutathione concentrations. In the absence of sulfhydryl groups, disulfide

Chart 4



bonds are thermodynamically stable and provide reasonably good stability in the bloodstream.

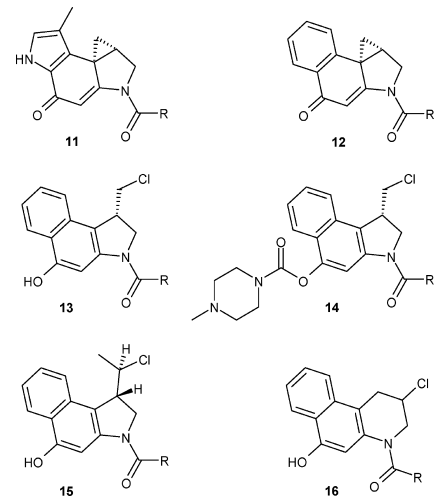
An example of an intracellularly cleavable disulfide-based linker was already discussed above (see "amide" conjugate **3**). Other examples are taxoid ADC payloads, conjugated via a disulfide-bearing 4-mercaptopentanoate linker to mAbs directed against the epidermal growth factor receptor (*19*). However, the most significant examples are ADCs where the immunoglobulin is connected over the same type of linker to ImmunoGen's drugs DM1 or DM4 (*20*−*22*). These potent thiol-containing maytansine analogues are antimitotic agents that bind to tubulin and inhibit microtubule assembly. After internalization of the ADC via antigen-mediated endocytosis and delivery to lysosomes by vesicular trafficking, the mAb is believed to be degraded, affording lysine derivatives linked to the toxin (*22*). Further intracellular modifications like cleavage of the disulfide linker through a disulfide exchange, and finally thiol methylation presumably catalyzed by intracellular methyltransferases, generate potent derivatives of DM1 or DM4 (*22*). These methylated drugs are uncharged, enabling them to pass back out through cell membranes to broaden their attack; the drug not only kills from inside the cell, but also knocks out adjacent cells within the tumor that are not targeted by the antibody (*23*). The linker of compound **4** (Chart 3) nevertheless proved to be relatively unstable *in vivo* (half-life of 47 h in mice) (*24*). Replacement of DM1 by DM4 structurally leads to the introduction of two methyl substituents on the carbon atom geminal to the disulfide bond, thereby limiting the accessibility for reducing agents. Consistent with these considerations, ADC **5** exhibits an increased half-life of 102 h in mice.

ENZYME-LABILE LINKERS

Chemically labile linkers often suffer from limited plasma stability. An alternative approach to achieve better control of the drug release involves linking a drug to an antibody via a peptide linkage. The free drug can be specifically cleaved from the carrier by the action of lysosomal proteases (e.g., cathepsin or plasmin) present at elevated levels in certain tumor tissues (*25*). Peptidic bonds are expected to have good serum stability, as proteases are normally not active outside cells because of unfavorable pH conditions and inhibition by serum protease inhibitors.

Seattle Genetics is using a maleimido-containing dipeptide linker to conjugate cysteine residues of mAbs with monomethyl auristatin E (MMAE, **7**) and F (MMAF), two auristatin derivatives that bind to tubulin and inhibit microtubule assembly. A valine-citrulline linker was designed to provide high plasma stability (half-lives in mice and monkey of 6.0 and 9.6 days, respectively) as well as a cleavage site for proteases (*26*). Efficient hydrolysis of the dipeptide linker by cathepsin B has been established *in vitro*. After enzymatic

Chart 5



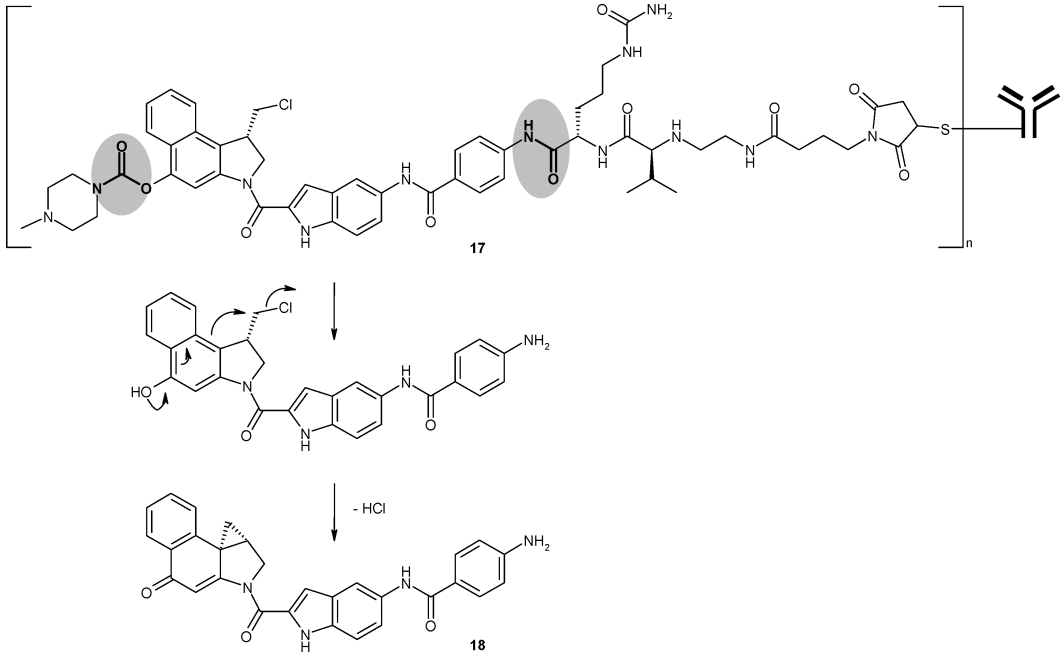
cleavage, 1,6-elimination of the strongly electron-donating 4-aminobenzyl group will occur, releasing MMAE in its active form (Scheme 1). This self-eliminating spacer is needed to spatially separate the drug from the site of enzymatic cleavage (*27*). Otherwise, the bulky payload has a negative influence on the kinetics of the peptide hydrolysis, as the access to the enzyme's active site can be affected.

Comparison of plasma stabilities of ADCs bearing enzyme-labile valine-citrulline or phenylalanine-lysine linkers (**6** and **8**, respectively) with ADC **9** equipped with a chemically labile hydrazone linker connecting MMAE with the cBR96 mAb showed impressively the advantage of the peptide linkage (Table 1): in human plasma, the ADC **6**, featuring a peptidic valine-citrulline-derived linker, exhibits an almost 100-fold increased stability compared to its hydrazone relative **9** and the *in vitro* specificity was increased (*26*). The peptidic linker has a half-life of 6 days in mice compared to 2 days for the hydrazone linker (3-fold improvement), resulting in a lower *in vivo* toxicity (*28*). Several ADCs incorporating a peptidic linker are in clinical development, with the most advanced being cAC10-vcMMAE (SGN-35) for CD30-positive hematologic malignancies (*26, 29, 30*).

In addition to *N*-terminus linked MMAE and MMAF, attachment of dipeptide linkers at the *C*-terminus was studied as a way to vary the rate of drug release and thus ADC potency and tolerability (*26, 31*). For MMAF, it could be shown that linking the mAb over a dipeptide linker to the C-terminus of the drug can lead to further improvement of the therapeutic window.

The superiority of enzyme-labile linkers was also shown for doxorubicin derivatives conjugated to mAbs (*11, 32*). With this

Scheme 2. Presumed Release Mechanism of CBI-Containing Drug 18 from ADC 17



strategy, the immunological specificity of BR96 mAb−peptide−doxorubicin conjugates **10a** and **10b** (Chart 4) was significantly improved compared to that of the corresponding hydrazone-based conjugate **1** (*11*), demonstrating again the importance of the linker structure for the ADC properties.

Peptide-containing linkers have been successfully used with CC-1065-derived toxins. CC-1065, duocarmycins, and other cyclopropapyrroloind-4-one (CPI, **11**; Chart 5) derivatives are potent minor-groove binding DNA alkylating agents. According to Boger et al., cyclopropabenzindol-4-one analogues (CBI, **12**) are chemically more stable, biologically more potent, and synthetically more accessible than their parent compounds incorporating the natural CPI alkylation subunit (*33*). In analogy to a duocarmycin prodrug (*34*), the seco-CBI precursor **13** was developed. This compound, which was postulated to show improved pharmacokinetics, will provide the CBI analogue **12** via Winstein cyclization (*35*). A further improvement was achieved by protecting the phenolic hydroxyl group with an enzyme-labile carbamate, as in derivative **14**, decreasing the toxicity of the prodrug while increasing its water solubility by introduction of an additional basic nitrogen (*36*).

Using their UltiMAb technology, Medarex conjugated compound **14** to a mAb via a peptidic linker (*37*). Conceptually, this approach is a double prodrug strategy. It does not only improve the drug's therapeutic efficacy by conjugating it to a tumor-specific mAb, but additionally employs a prodrug of the cytotoxic agent that has reduced potency and thus minimizes side effects before being released intracellularly by carboxyesterases. The two activating steps needed to trigger the release of the active drug (Scheme 2) may lead to an enlarged therapeutic window.

Tietze et al. have reported that the CBI prodrugs **15** and **16** exhibit lower toxicities due to the reduced reactivity of the secondary chloride compared to the primary chloride present in **13** and **14** (*39−41*). Such derivatives with secondary chlorides (see, for example, ADC **19**; Chart 6) are used in Syntarga's SpaceLink conjugation technology (*42*). To our knowledge, no data are yet published on the linker stability.

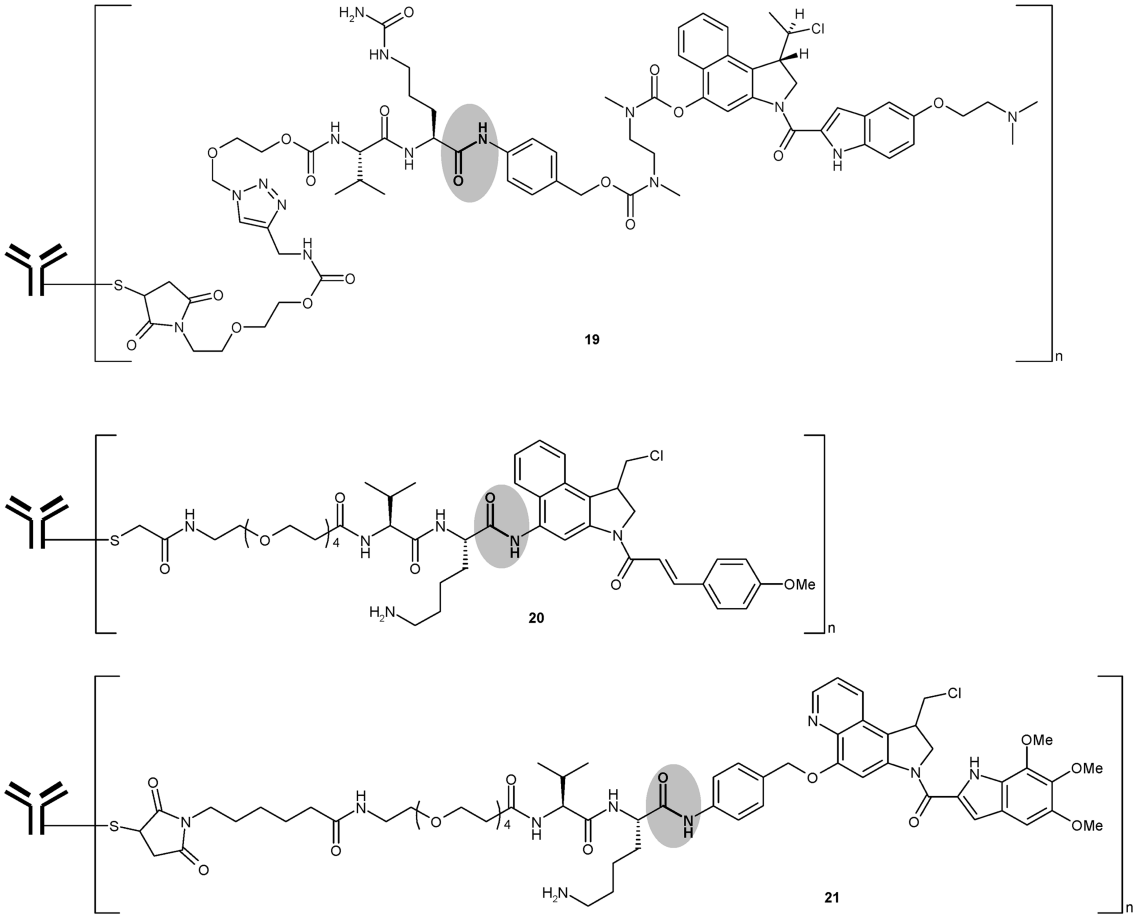
To extend its ADC technology to drugs with a complementary mode of action, Seattle Genetics has developed ADCs **20** and **21** (Chart 6) containing an amino-CBI and a hydroxy aza-CBI payload, respectively (*43*). Because of the hydrophobicity of this class of molecules, attention was focused on developing hydrophilic peptide-linker derivatives that prevented aggregation. This was achieved by use of a more hydrophilic valine-lysine sequence (compared to valine-citrulline as in ADC **6**) and incorporating a tetra(ethylene glycol) unit between the mAb and the peptide linker. The direct attachment of the linker to the amine of the CBI building block (as in **20**) or over a self-immolative *p*-aminobenzyl spacer to the corresponding hydroxyl group of the aza-CBI toxin (as in **21**) prohibits the spontaneous formation of the active toxin in the plasma. Only the enzymatic cleavage of the linker after internalization of the ADC into the cancer cell triggers the release of the prodrug that can now be transformed into the DNA-alkylating cyclopropyl derivative via a Winstein cyclization.

Another type of enzyme-labile linker was recently reported by Seattle Genetics (*44*). In this case, a carbohydrate moiety serves as the cleavage site. Its hydrophilic nature should limit aggregation. Interestingly, the β-glucoronide moiety in ADC **22** is not directly linking the ADC with the payload. Nevertheless, cleavage by β-glucuronidase triggers the 1,6-elimination of the spacer liberating the free drug **23** (Scheme 3). This carbohydrate motif was also used in ADCs bearing Camptothecin analogues (*45*).

NONCLEAVABLE LINKERS

ImmunoGen has reported the fortuitous discovery of a noncleavable thioether bond to link DM1 to mAbs (*22, 46*). Derivative **24** was actually prepared as a control experiment, but surprisingly, this noncleavable thioether linked ADC also proved very potent. The release mechanism is believed to occur via internalization of the ADC followed by degradation of the mAb component in the lysosome, resulting in the release of the maytansinoid drug **25** still attached via the linker to a lysine residue. This chemical modification of the drug did not diminish its cytotoxic potential. This form of the drug is, however, charged and presumably not able to diffuse into neighboring cells. Hence, it cannot kill adjacent tumor cells

Chart 6

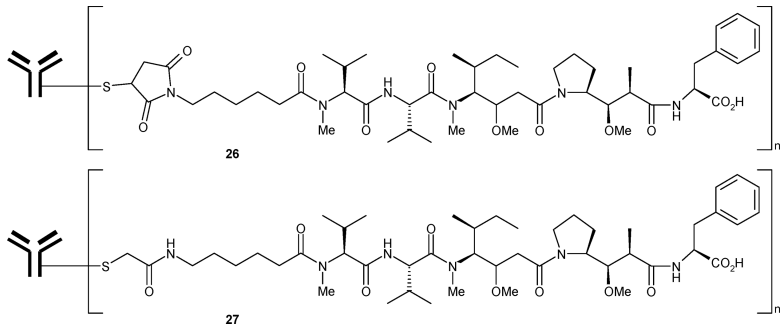


that do not express the target antigen (antigen-negative cells). Besides this by-stander effect (*23*), there is an additional reason why cleavable linkers may have a broader efficacy. Although internalization is often the common initial activation process for both cleavable and noncleavable linkers (*22*), ADCs with cleavable linkers may also be active even when they are poorly internalized (*47*). One possible explanation is that, when the linker is cleaved in extracellular space, the free drug subsequently permeates the cell to reach its target. This mechanism is not operating for ADCs incorporating noncleavable linkers, which must be internalized and degraded within the cell to become active. They are thus dependent on the biology of the target cell.

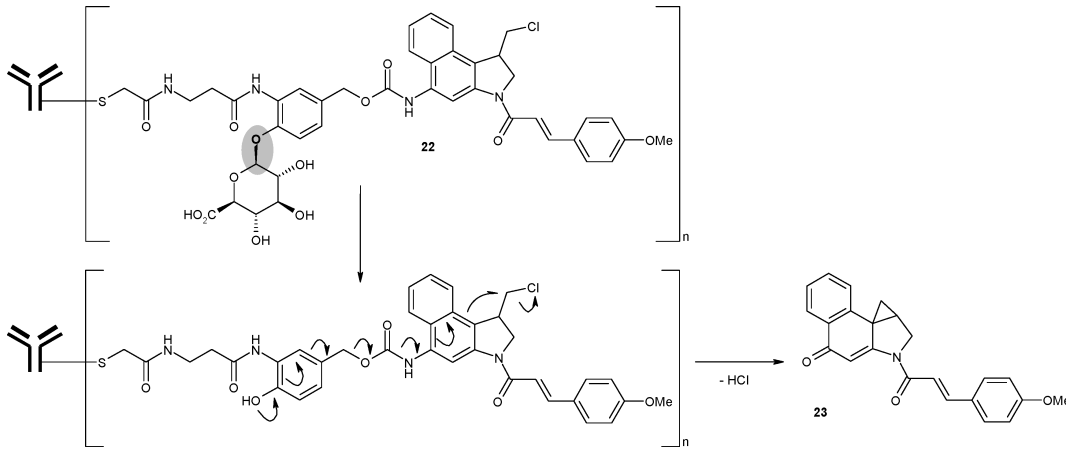
The *in vivo* stability of the thioether-linked ADC **24** proved, with a half-life of 134 h in mice, superior to that of compounds **4** and **5** (47 and 102 h, respectively). ADCs with noncleavable linkers were found to be better tolerated (*47*), presumably resulting in an improved therapeutic index. The use of noncleavable linkers has thus become an important feature of ImmunoGen TAP (tumor-activated prodrug) conjugation technology. This is best exemplified by the promising clinical results obtained by Genentech's trastuzumab-DM1 (T-DM1) for HER2-positive metastatic breast cancer (*48*).

Seattle Genetics made the same finding with MMAF, namely, that the cleavable dipeptide linker (as in **6**) could be replaced by a noncleavable thioether bond (as in ADC **26**; Chart 7) while retaining the *in vivo* activity (*49*). Mass spectrometry showed that the released drug was a cysteine-adduct of MMAF, presumably resulting from mAb degradation within lysosomes as proposed for maytansinoid conjugate **24**. MMAE, although closely related structurally, was not

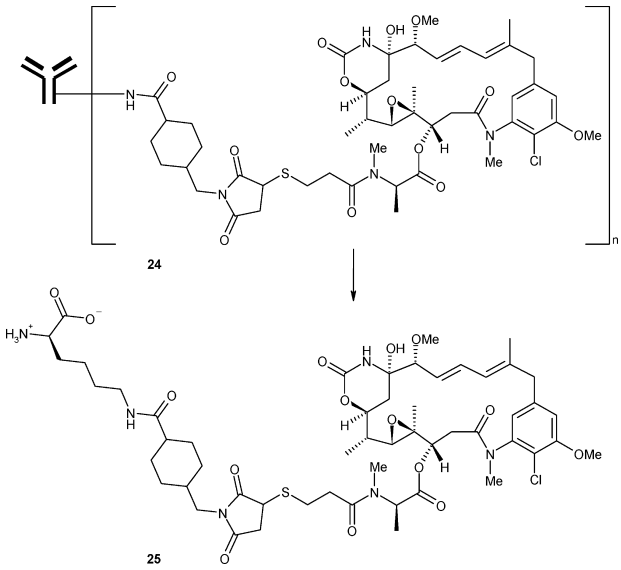
Chart 7



Scheme 3. Postulated Release Mechanism of CBI-Containing Drug 23 from ADC 22



Scheme 4. Release of Maytansine Drug-Derivative 25 through Lysosomal Degradation of ADC 24



active when attached in this manner. Only MMAF can sustain significant modification to the *N*-terminal position and still retain its potency. Recently, it was shown that a slow transfer of the drug from the ADC to albumin cysteines in the plasma occurs with alkyl-maleimide ADCs (*50*). To counteract this effect, the linker stability was enhanced by replacing the maleimide (as in ADC **26**) with an acetamide functionality (as in ADC **27**). This type of ADC led to no apparent degradation in the bloodstream for up to 14 days.

CONCLUSION

A promising approach to improve the potency of antibody-based cancer therapies is to conjugate the mAb with cytotoxic chemotherapeutic drugs. In the foreseeable future, ADCs will eventually fulfill the promise of specific delivery of cytotoxic drugs to tumor cells (targeted therapy), thus avoiding the dose-limiting toxicity of chemotherapy that occurs as a result of its effects on normal cells. The linker technology profoundly impacts ADC potency, specificity, and safety. Early ADC linkers were commonly derived from acid-labile hydrazones, which were designed to be cleaved inside target cancer cells, but inevitably underwent cleavage at nontarget sites. Newer linkers feature hindered disulfides and peptidic moieties that have achieved greater *in vivo* stability. To spare nontarget tissues from chemotherapeutic damage, noncleavable linkers were recently developed. They are thus far the most stable linkers used in ADCs. This recent development may shift some of the future challenge in ADC development from the linker technology toward finding cytotoxic drugs that retain their potency when bearing a covalently attached linker. The improved linker stability and reduced toxicity, however, come together with greater target dependence than in the case of cleavable linkers. ADCs with noncleavable linkers must be internalized and degraded within the cell, whereas compounds with cleavable linkers may be active against targets that are poorly internalized through extracellular drug release and drug entry into tumor cells. Similarly, killing of bystander antigen-negative cells through targeting of antigen-positive cells (collateral toxicity) is presumably only possible with cleavable linkers. The Lonza ADC-team thus believes that no general linker design exists for all ADCs or cancer types. Despite significant progress, each mAb must be examined separately in order to optimize a specific delivery system.

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