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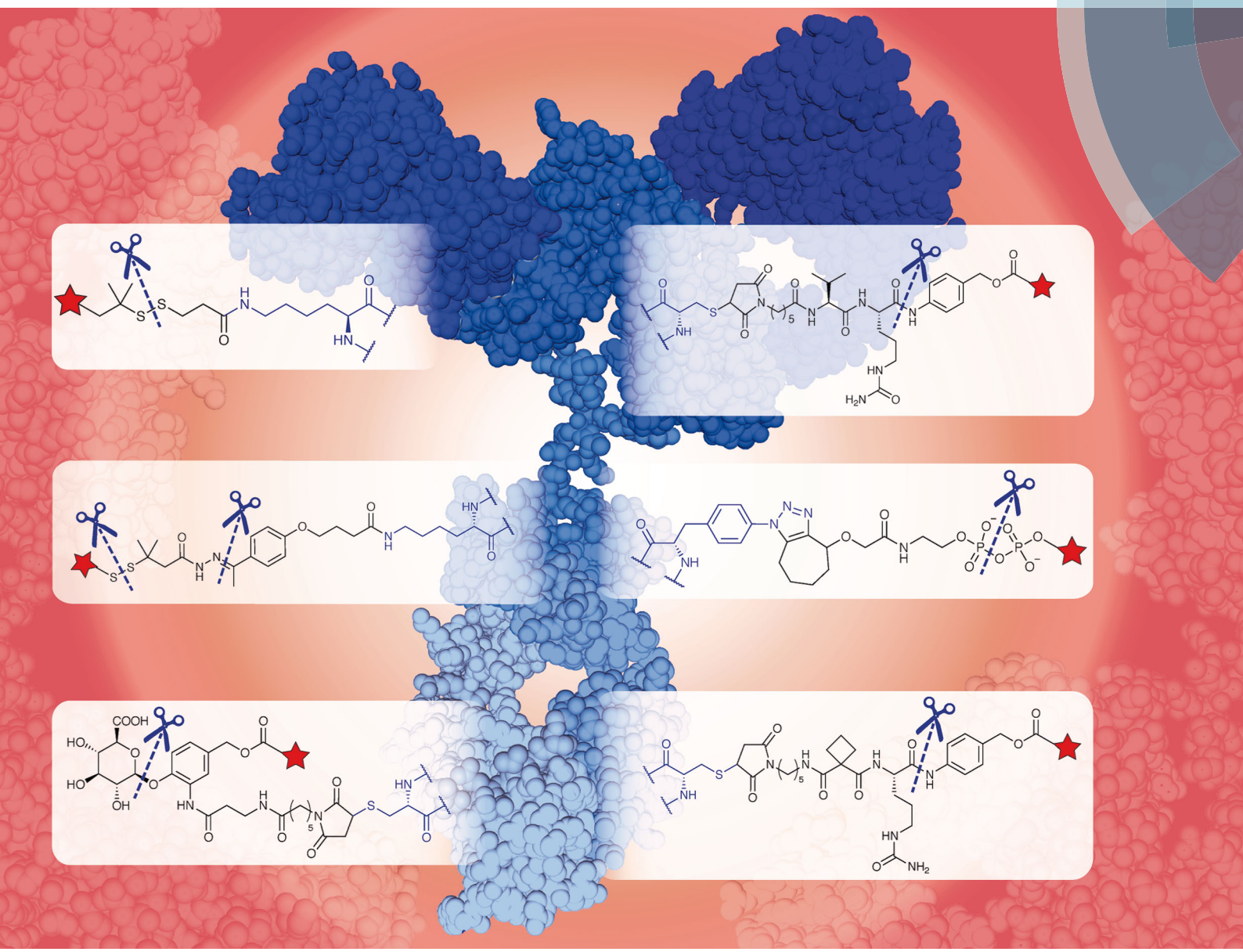

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TUTORIAL REVIEW

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Cleavable linkers in antibody–drug conjugates

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Antibody–Drug Conjugates (ADCs) are now established as a major class of therapeutics for the clinical treatment of cancer. The properties of the linker between the antibody and the payload are proven to be critical to the success of an ADC. Although ADC linkers can be 'non-cleavable', the vast majority of ADCs in clinical development have specific release mechanisms to allow controlled linker cleavage at the target site and are thus termed 'cleavable'. In recent years, the development of new methods of drug release from ADCs has continued in parallel to the deepening understanding of the biological processes underlying the mechanisms of action of pre-existing technologies. This review summarises the advances in the field of cleavable linker technologies for ADCs.

Key learning points

1. ADCs employing cleavable linkers are applicable to a wide range of cancer types due to their ability to release unmodified, diffusible payloads.
2. The use of highly cytotoxic ADC payloads places stringent stability requirements upon the linker.
3. Linker stability is highly dependent on the attachment site on the antibody.
4. Disulfides and dipeptides are the dominant motifs found in cleavable linkers in the clinic and they continue to be improved.
5. Extracellular drug release at the tumour site alleviates the requirement for internalising antigens, opening the door to a wider range of target antigens.

1. Introduction

Antibody–Drug Conjugates (ADCs) are a rapidly growing class of targeted drug delivery therapeutics comprising a monoclonal antibody (mAb) connected *via* a covalent linker to a small molecule cytotoxic payload. There are currently four approved ADC medicines (Fig. 1) and over 50 are in clinical trials.[1] Whilst ADCs can in principle be applied to the treatment of a range of diseases, research thus far has overwhelmingly focussed on oncology. Small molecular weight cytotoxins have historically been the most commonly employed chemotherapy agents due to their high cytotoxicity and relatively low production costs. However, they suffer from low selectivity towards cancer cells and rapid plasma clearance ($t_{1/2}$ = hours). Conversely, modern mAb therapies can target cancer cells with high selectivity by recognising and binding to cell-surface antigens that are overexpressed in certain cancerous indications. Furthermore, mAbs can also exhibit long plasma half-lives ($t_{1/2}$ = weeks), but they have limited cytotoxicity.[2] ADCs therefore aim to combine the favourable aspects of both therapies, creating highly cytotoxic and selective therapeutics with long plasma half-lives.

The traditional mechanism of action of an ADC involves antibody–antigen binding on the target cell surface, internalisation by endocytosis and lysosomal processing to release the cytotoxic payload (Fig. 2A).[3] In some cases, the payload may be sufficiently membrane-permeable to diffuse out of the cell and kill bystander tumour cells. By this 'bystander effect', non-antigen expressing cancer cells in a tumour can also be targeted.[4]

Although the vast majority of ADC research thus far has focussed on the traditional mechanism of action, a non-internalising mechanism of action is also possible. In this case, linker cleavage and payload release occurs in the extracellular tumour microenvironment (Fig. 2B).[5] Thus, ADC endocytosis is not required and non-internalising antigens may be selected as targets. The benefits of this mechanism of action include a much wider selection of possible target antigens, avoidance of the potentially inefficient intracellular trafficking process and less dependence on high cell-surface antigen expression. However, the employment of extracellular stimuli may also lead to lower cell-selectivity and off-target toxicity.

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For ADCs to be selective and potent, the linker technology employed should strive towards three key properties:

(1) High stability in circulation.

(2) High water solubility to aid bioconjugation and avoid formation of inactive ADC aggregates.

(3) Allow efficient release of a highly cytotoxic payload-linker metabolite.

Linkers comprise two key parts: the antibody- and the payload-attachment (Fig. 1). The bonding between the linker and the mAb is critical since it defines the drug-to-antibody-ratio (DAR) and therefore the homogeneity and stability of the ADC. Bioconjugation methods for ADCs are a hot topic of research and have been reviewed extensively elsewhere.[6] The bonding between the linker and the payload is equally important and will be the subject of this review.

ADC linkers can be classified as 'cleavable' or 'non-cleavable'. For non-cleavable linkers, there is no inbuilt chemical trigger to cleave the linker. Upon entry to the lysosome, the mAb is metabolised by proteolytic machinery into its constituent amino acids. The payload that is released therefore contains the drug, the linker and an amino acid appendage. Surprisingly, this drastic modification to a payload can result in efficacious ADCs if the key pharmacophore of the payload is unaffected, as in the case of Kadcyla® (Fig. 1B).[7] However, non-cleavable linkers are generally unable to exert a bystander effect due to the lack of cell permeability with the charged amino acid appendage. Non-cleavable linkers are therefore mainly effective for the treatment of haematological cancers or tumours with high antigen expression, since over 99% of tumour cells must be killed to achieve remission in a patient.[2]

Conversely, cleavable linkers exploit specific conditions to release the drug at the target cell. For instance, endocytosis leads ADCs to the lysosome, a unique compartment with a low pH and high concentration of hydrolytic enzymes. The cytosol of the cell is

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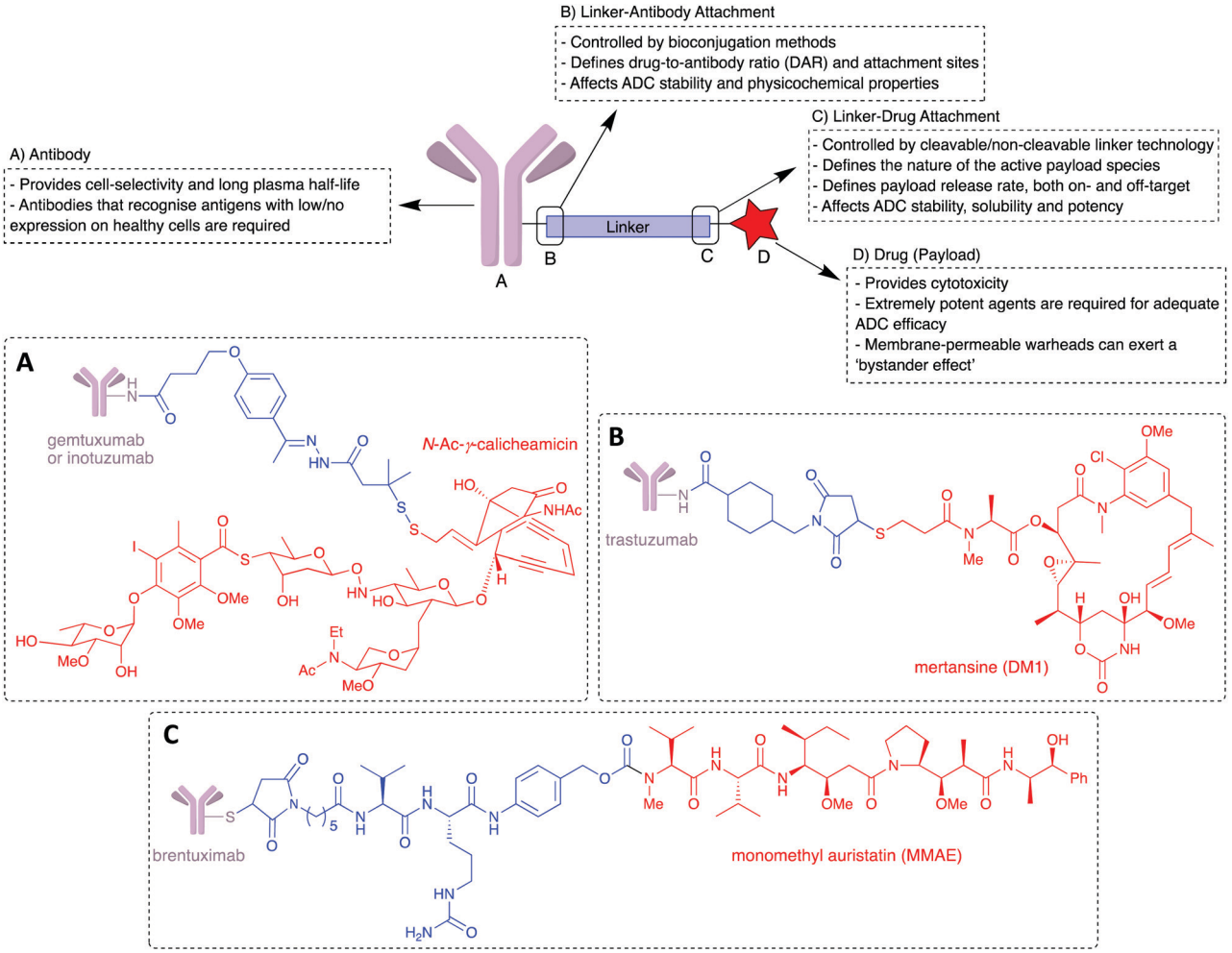

Fig. 1 (top) The general structure of an ADC and the role of each component. (bottom) The chemical structures of the four currently FDA-approved ADCs with the linkers in blue and the payloads in red. (A) gemtuzumab ozogamicin (Mylotarg®) and inotuzumab ozogamicin (Besponsa®) (B) trastuzumab emtansine (Kadcyla®) (C) brentuximab vedotin (Adcetris®).

also differentiated from extracellular conditions due to its high concentration of glutathione. Alternatively, ADC linkers can also be labile to the extracellular tumour microenvironment, according to the non-internalising mechanism of action previously discussed.

Cleavable linkers can be further subcategorised as chemically-cleavable or enzyme-cleavable. Both approaches have their merits, evidenced by three of the four approved ADCs containing cleavable linkers, as well as the vast majority of ADCs currently in clinical trials.[8]

Although cleavable linkers are generally preferred to non-cleavable linkers due to their range of applicability, there is a greater potential for instability in circulation. The success of cleavable linkers therefore depends on their ability to effectively differentiate between circulatory and target-cell conditions. The poor tumour penetration of large IgG antibodies, combined with any internalisation, intracellular trafficking and drug release inefficiencies, leads to the requirement of robust cleavable linker methodologies that maximise delivery of the potent cytotoxins to cancer cells.

The stability of ADC linkers are not only defined by the cleavable linker technology but also the attachment site on the antibody. Conjugation to a large antibody renders linkers less accessible to chemical and enzymatic triggers, thereby increasing plasma stability and slowing target cell release rates. This effect can be further amplified when site-selective bioconjugation techniques are employed to attach the linker-payload to less solvent-accessible sites.[9,10]

2. Chemically cleavable linkers

There are three main types of chemically cleavable linkers: acid cleavable, reducible disulfides and those cleavable by exogenous stimuli. The first two are clinically established methods, since two of the four currently marketed ADCs contain acid cleavable linkers and reducible disulfides constitute the largest class of chemically cleavable ADC linkers in clinical trials.

2.1. Acid cleavable linkers

Acid cleavable linkers aim to exploit the acidity of the endosomes (pH 5.5–6.2) and lysosomes (pH 4.5–5.0), whilst maintaining

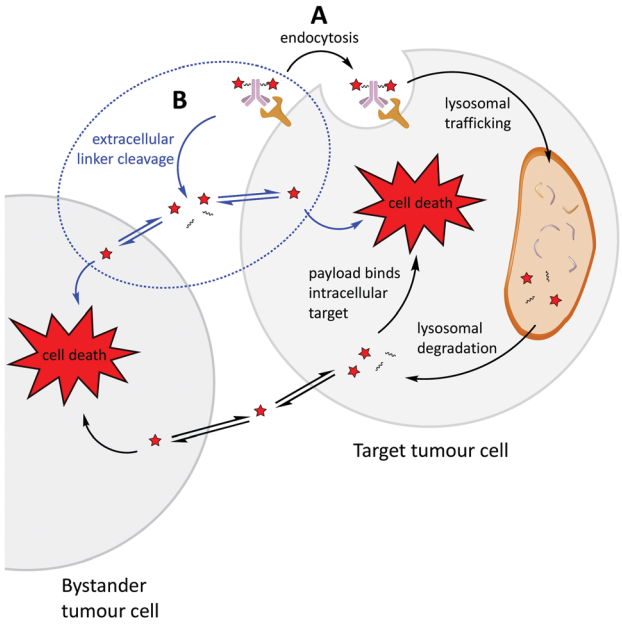

Fig. 2 (A) The traditional mechanism of action, involving endocytosis and intracellular payload release. (B) The non-internalising, extracellular mechanism of action.

stability in circulation at pH 7.4. This strategy yielded the earliest clinical success in the field with Pfizer's gemtuzumab ozogamicin (Mylotarg®) ADC (Fig. 1A).[11] Whilst also employing a reducible disulfide, the linker contains an acid-sensitive *N*-acyl hydrazone linkage that, upon acid catalysis, hydrolyses to a ketone and a hydrazide-payload. In its development, the stabilities of a range of hydrazones were tested at pH 4.5 and pH 7.4. These hydrazone linkers were tested in ADCs *in vitro* and *in vivo* in mice and those that were stable at pH 7.4 and labile at pH 4.5 afforded the most potent and efficacious ADCs.

This linker-payload combination is also employed in inotuzumab ozogamicin (Besponsa®), approved in 2017. Analysis of the *in vivo* stability of the Besponsa® linker showed that hydrazone hydrolysis occurred in circulation at a rate of only 1.5–2% per day.[12] However, other hydrazone-containing ADC linkers have demonstrated discrepancies between buffer stability and plasma stability. Notably, a phenylketone-derived hydrazone linker was hydrolysed with $t_{1/2}$ = 2 days in isolated human and mouse plasma, despite a much higher stability profile in pH 7.4 buffer (Fig. 3).[13] The specific cause of the increased hydrolysis rate in plasma remains unclear but the highly variable stability profile of hydrazones compared to other cleavable linkers has hampered their utility.

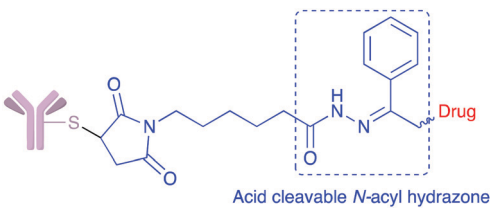

Fig. 3 The structure of a phenylketone-derived hydrazone linker.

Whilst the concept of acid-cleavable linkers to release payloads extracellularly has not been considerably developed, proof-of-concept has been achieved in a number of cases. The hydrazone-containing linker-payload from Mylotarg® was conjugated to rituximab, a non-internalising mAb.[14] Despite the non-internalising nature of the mAb, the ADC showed superior efficacy to free rituximab in a mouse xenograft model. Furthermore, a non-cleavable analogue was less active, proving the necessity of acid-hydrolysis of the hydrazone for efficacy.

Acid cleavable linkers containing other functional groups have also been reported, such as the combination of carbonate linkers with an alcohol-containing SN-38 payload (Fig. 4).[15] Whilst ester linkages were expected to be more stable in circulation than carbonates, an ADC linked by the former was too labile in isolated human serum. Although simple alkyl-carbonate linkages were only marginally improved, introduction of a *para*-aminobenzyl (PAB)-spacer boosted the serum stability of the ADC to an acceptable $t_{1/2}$ = 36 h. The more stable carbonate demonstrated some selectivity to the acidic lysosomal compartment, with a $t_{1/2}$ = 10 h at pH 5.[16] This linker technology was applied to two different mAbs in ADCs that progressed to Phase I/II clinical trials. In the case of a CD74-targeting mAb, the ADC was efficacious *in vivo* in mice despite the relatively low cell-surface expression of CD74. Furthermore, when applied to mAbs targeting poorly internalising antigens, the ADCs were also efficacious, suggesting that a non-internalising release may be the dominant mechanism of payload release.

Most ADC linker technology has moved away from acid-cleavable groups, despite the clinical success of Mylotarg® and Besponsa®. The requirement for linkers to strictly discriminate between pH 5 and pH 7.4 is inherently difficult and development now focuses on other approaches that can yield higher tumour selectivity. Whilst in some cases, slow circulatory payload release has yielded promising results in mouse models, this approach has generally employed only moderately cytotoxic payloads. The highly potent payloads that are now preferred for ADCs require more stable linkers.[13]

2.2. Reducible disulfides

Disulfides are the most prominent class of chemically cleavable motifs found in ADC linkers and, alongside hydrazones, they have found clinical success in Pfizer's Mylotarg® and Besponsa® (Fig. 1A). Disulfides are stable at physiologic pH but are susceptible to nucleophilic attack from thiols (Fig. 5). In blood plasma, the dominant thiol species is the reduced form of human serum albumin (HSA) (~422 μM), however its reactivity towards large molecules is hampered because the free thiol-containing residue

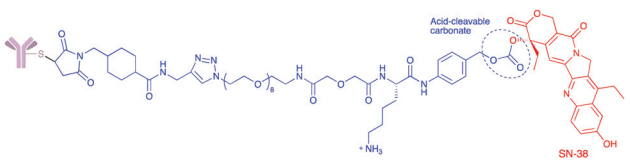

Fig. 4 The structure of SN-38-bearing ADCs with an acid-cleavable PAB-carbonate linker.

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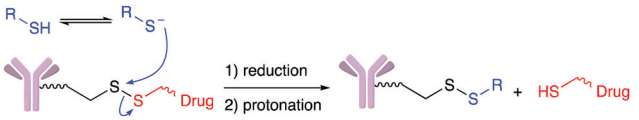

Fig. 5 The reduction of disulfide linkers, mediated by the deprotonated thiolate.

(Cys34), is located near a crevice with limited solvent exposure.[17] In contrast to the limited reductive power of blood plasma, the cytosol contains high levels of glutathione (GSH) (1–10 mM), a thiol-containing small molecule tripeptide.[17] This large discrepancy between the reductive potential of blood plasma and the cytosol offers an opportunity for selective intracellular payload release from ADCs. Furthermore, the oxidative stress associated with tumours often leads to elevated GSH levels compared to healthy tissue, which adds an extra level of selectivity towards cancer cells. Intracellular protein disulfide-isomerase (PDI) enzymes may also aid disulfide reduction of ADC linkers, although this contribution has not been verified.

Despite the clinical success of Pfizer's calicheamicin-ADCs, disulfide-containing ADC linkers have been mainly paired with the maytansinoid class of payloads, originally developed by Immunogen in 1992.[18] Maytansinoids are an extremely potent class of tubulin-binding cytotoxins derived from the natural product maytansine (Fig. 6). Maytansinoids with a thiol appendage β- to the amide are attractive derivatives due to the ease of conjugation and high potency.

2.2.1. Sterically hindered disulfides. In 2008, researchers at Genentech and Immunogen investigated the effects of α-methyl substitution on disulfide linker stability with ADCs targeting HER2+ breast cancer (Fig. 7). By increasing steric protection around the disulfide, the linkers became less susceptible towards reduction.[9] The unsubstituted linker's stability profile was incompatible with maytansinoid-ADCs, with full release of the payload *in vivo* in mice after three days. Both the unsubstituted linker and the most substituted linker showed poor antitumour activities whereas linkers with intermediate levels of steric-substitution were the most active, suggesting a balancing act between extracellular stability and intracellular cleavage efficiency. The linker with one methyl-group on each side of the disulfide (R^1 = H, R^2 = Me, drug = DM1, Fig. 7) was

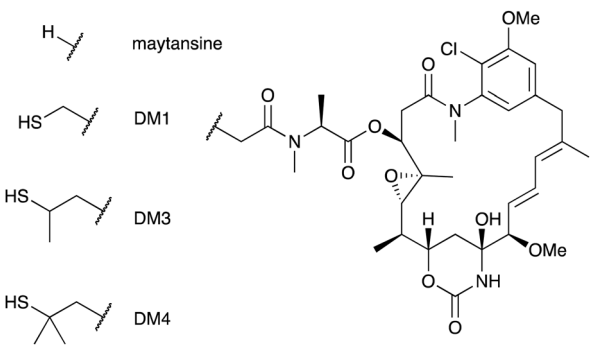

Fig. 6 The structures of maytansine and the maytansinoids DM1, DM3 and DM4.

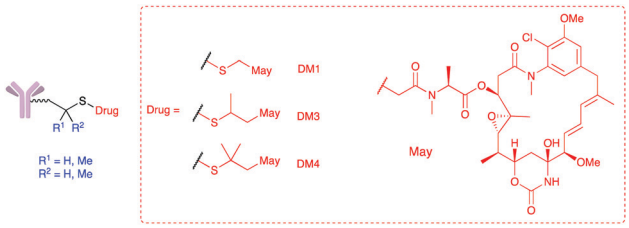

Fig. 7 Maytansinoid-ADCs with varying steric protection around the disulfide bond.

the most active, alongside a non-cleavable analogue. A similar stability pattern was observed in the case of maytansinoid-ADCs targeting the antigen CanAg.[19] For this cancer type, the linker containing two methyl groups on the payload side of the disulfide yielded the most active ADC in both homogeneous and heterogeneous antigen-expressing xenografts. The superiority of this di-unsubstituted:DM4 linker-payload (R^1 = R^2 = H, Drug = DM4, Fig. 7) over dimethyl:DM1 (R^1 = R^2 = Me, Drug = DM1, Fig. 7) arises not from the stability profile but instead the increased activity of the DM4 metabolite (*vide infra*). The most hindered disulfide (three methyl groups) and the non-cleavable linker both showed poor activity *in vivo*, illustrating the importance of efficient drug release for this tumour type. Taken together, these findings suggest the optimal properties of an ADC linker are unique to each cancer and one size does not fit all.

In both of these studies, the *in vitro* potency was unaffected by any linker changes whereas the *in vivo* antitumour activities were dramatically different. However, the bystander activity of the anti-CanAg ADCs was investigated by measuring their cytotoxicity against antigen-negative cells, co-cultured alongside antigen-positive cells.[19] Using this technique, *in vitro* potency against the co-culture did accurately reflect the *in vivo* activity. Clearly for some cancer types the bystander effect is a critical component of antitumour activity and *in vitro* potency of isolated cells may not be a good indicator of efficacy.

2.2.2. Intracellular disulfide metabolism. The metabolism of disulfide-linked ADCs has been investigated, with both DM1 and DM4 payloads linked to anti-HER2 and anti-CanAg mAbs.[20,21] For these ADCs, disulfide reduction only occurs following mAb degradation in the lysosome, thereby necessitating efficient lysosomal processing for antitumour activity (Fig. 8). For these lysine-conjugated ADCs, the mAb is degraded into its constituent amino acids, thereby releasing the linker-payload with a lysine appendage. This sterically unhindered lysine-linker-payload metabolite is then readily cleaved by small molecule reductants in the cytosol. In the case of a DM4 payload, rapid *S*-methylation occurs to form the potent thioether derivative, presumably by an intracellular methyltransferase. Conversely, the DM1 payload is not significantly *S*-methylated after eight hours, which may partly explain the inferior bystander effect of the DM1-containing ADCs targeting CanAg.[19,21]

2.2.3. Disulfides formed with antibody cysteine residues. Recently, direct disulfide-bonding between engineered cysteine residues and maytansinoid thiols have been utilised in ADCs (Fig. 9A).[17] By shielding the disulfides from reduction using the

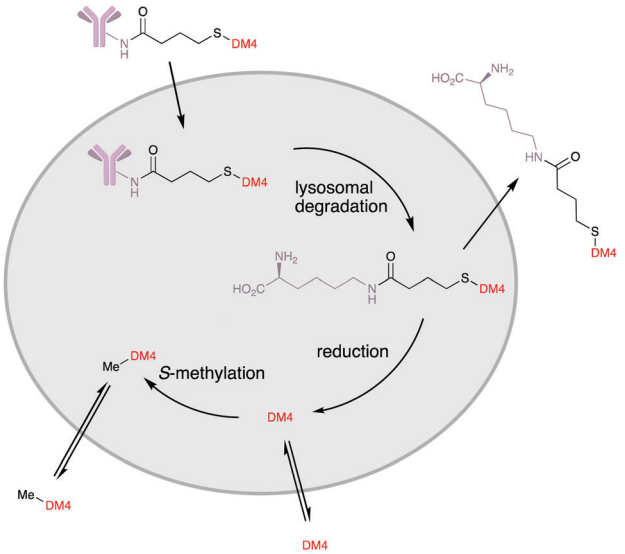

Fig. 8 Intracellular metabolism of a CanAg-DM4 ADC.

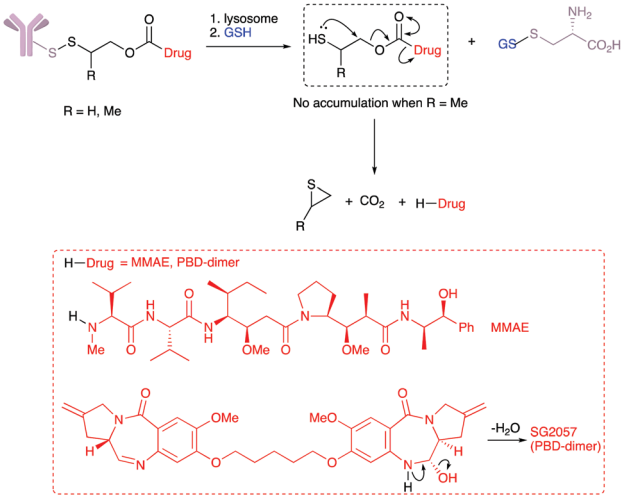

Fig. 10 Disulfide reduction and immolation of a disulfide-carbamate, releasing amine-linked drugs.

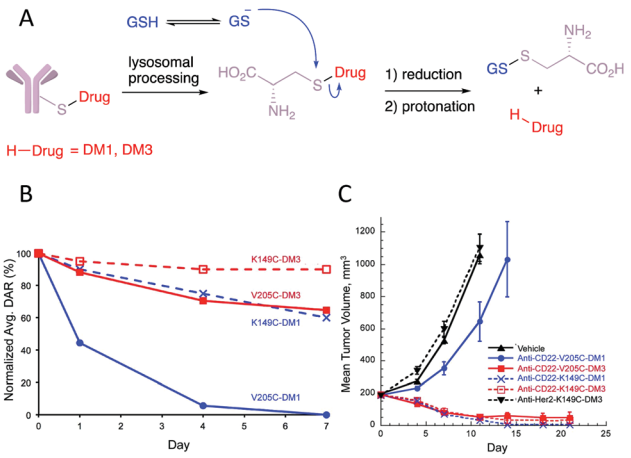

Fig. 9 (A) Metabolism of a linkerless, cysteine-linked maytansinoid ADC (B) *in vivo* stability of the conjugates in healthy mice dosed at 3 mg kg^{-1} (C) antitumour activity of the conjugates in a CD22-xenograft model, dosed at 3 mg kg^{-1}. K149C and V205C refer to the hindered and unhindered attachment sites respectively. Reproduced from ref. 17 with permission from the Royal Society of Chemistry.

antibody, researchers at Genentech achieved excellent *in vivo* mouse plasma stability without the need for excessive steric protection around the payloads. The stability of the resulting disulfides was highly site-dependent, with less solvent-exposed sites exhibiting dramatically increased linker stability (Fig. 9B). The sterically protected DM3 payload gave the predicted rise in stability over DM1, whereby only 10% was cleaved after seven days in the hindered conjugation site. The increase in stability, from conjugation site and/or methyl substitution was reflected in a remarkable increase in antitumour activity in mice (Fig. 9C). Comparison of the linkerless ADC with ADCs employing known linker-DM4 motifs revealed the superior efficacy of the linkerless ADC at 3 mg kg^{-1}. The authors posit that the increased efficacy can be attributed to the decoupling of plasma stability and payload release efficiency, whereby high plasma stability and rapid drug release can be achieved concurrently. Furthermore, a novel self-immolating carbamate linker was employed to allow the efficient release of an MMAE payload, linked by its secondary amine functionality (Fig. 10). This technology allows the benefits of disulfide technology to be applied to a wider range of potential payloads.

Further exploring this traceless disulfide-carbamate technology, researchers at Genentech attached highly potent pyrrolobenzodiazepine (PBD)-dimer payloads to ADCs through their secondary amine functionality (Fig. 10).[22] Alongside increasing stability towards reduction, an α-methyl group also increased the rate of self-immolation to release the native payload, a step required for cytotoxicity (Fig. 10). With this ADC, full tumour inhibition was achieved at dosing as low as 1 mg kg^{-1}. This remarkably simple linker system performed comparably *in vivo* with a more complex protease cleavable linker bearing the same payload, suggesting the cysteine–disulfide platform is well suited to releasing a variety of ADC payloads.

2.2.3.1. Extracellular disulfide reduction. Whilst the traditionally viewed mechanism of action for ADCs requires internalisation, lysosomal trafficking and subsequent payload release, the Neri laboratory has demonstrated that non-internalising ADCs with cleavable disulfide linkers can be efficacious.[23] Extracellular drug release was achieved at the tumour in mice by using a tumour vasculature-targeting antibody with linkerless disulfide technology. The tumour-selective drug release is caused by the release of high concentrations of reductants from dying tumour cells into the extracellular space, coupled with an insufficient oxygen supply (hypoxia) often associated with tumours. The initial payload release and subsequent cell death, thereby causes a 'chain reaction', where each dying cell causes a growing concentration of reductants within the tumour.

The researchers used a tumour-targeting antibody F8, which targets the alternatively spliced Extra Domain A (EDA)

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of fibronectin and rapidly accumulates in tumours. The antibody was expressed in small immune protein (SIP) form, allowing superior tumour targeting and fast clearance in circulation, thereby minimising off-target payload release. A thio-auristatin payload was linked to the SIP *via* a disulfide bridged to two engineered cysteines on the C-termini. When the ADC was applied to a xenograft model, it outperformed the free payload and an analogous ADC with a non-specific antibody. However, a very high dose of 43 mg kg^{-1} was required to achieve substantial prolongation of survival and none of the five mice were cured. Subsequently, the group was able to achieve 3/5 cures in mice at 7 mg kg^{-1} by employing a more potent DM1 payload.[24] Gratifyingly, the ADCs were well tolerated, signifying that off-target DM1 release is suitably low.

When the DM1-containing ADC that employed the SIP antibody was compared to an analogous IgG ADC, payload release was much faster in the SIP-containing ADC.[25] The authors attributed this to the favourable rearrangement of disulfide structures in the SIP, leading to a stable interchain disulfide bond. When the stability of the ADCs were analysed in isolated mouse plasma, $t_{1/2} > 48$ hours and <3 hours were observed for the IgG and SIP respectively. In an efficacy study, the SIP-containing ADC outperformed the IgG-ADC, suggesting that rapid tumour accumulation and payload release imparts greater antitumour activity for this cancer type. This surprising result is despite the overall tumour accumulation from the IgG ADC being higher after 24 hours.

2.3. Cleavage by exogenous stimuli

Whilst the cleavage of ADC linkers with endogenous triggers is the simplest method for drug release, external small molecule triggers of extracellular drug release may offer the following advantages: (1) avoidance of any disparity in linker cleavage rates caused by variable biology across patients, and (2) the ADCs can be effective when extracellular concentrations of endogenous triggers are insufficient for efficient payload release.

Stenton *et al.* have described a thioether-containing linker that, upon exposure to Pd^0, releases an amine-linked payload (Fig. 11).[26] The technology was applied to a HER2-selective nanobody, which offers rapid tumour accumulation and superior tissue penetration to an IgG antibody. When tested in HER2+ cells, the ADC was less potent than Dox on its own but equally potent in the presence of the $[Pd(COD)Cl_2]$ decaging reagent.

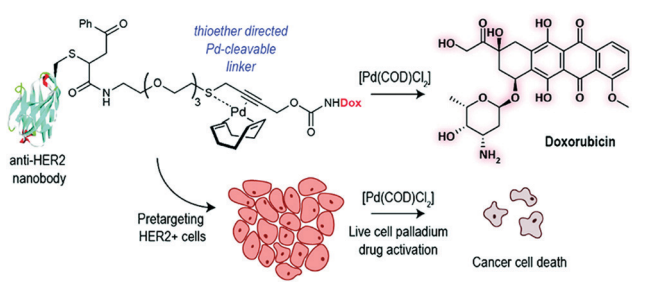

Fig. 11 Mechanism of action of a Pd^0-labile ADC. Reproduced with permission from ref. 26 with permission from the Royal Society of Chemistry.

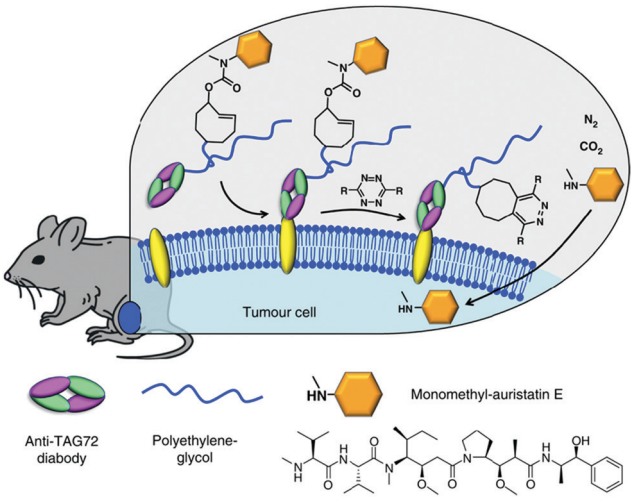

Fig. 12 The mechanism of action of a *trans*-cyclooctene-containing ADC, cleaved by a IEDDA reaction. Reproduced from ref. 28, used under license CC BY.

Although the concentration of Pd required for efficient decaging was non-toxic in kidney cells it is unlikely to be tolerated *in vivo*. Therefore more benign Pd complexes still need to be developed for this technology to be feasible for cancer treatment.

Rossin *et al.* have recently developed an ADC bearing an antibody fragment and a *trans*-cyclooctene linker that, upon inverse-electron demand Diels–Alder (IEDDA) reaction with an externally administered tetrazine, releases an amine-containing payload *in vivo* (Fig. 12).[27] The strategic placement of a carbamate at the allylic position causes elimination of CO_2 and an amine, and the resulting pyridazine rearranges to a pyridazone. Unfortunately the tetrazines with fast kinetic profiles were unsuitable for use on their own, due to their rapid plasma clearance rates ($t_{1/2} \approx 1$ min). The researchers were able to partially mitigate this problem by employing a pegylated 3-methyl-6-trimethylene-tetrazine linked to a chelated lutetium(III) species, which increased the activator half-life to 12 min.[28] The new activator was able to release 80–90% of the payload from the ADC in serum after 20 h of co-incubation, a drastic improvement over previous results with a dextran-containing tetrazine.[27] However, instability of the *trans*-cyclooctene–Dox conjugate towards alkene isomerism was observed, with $t_{1/2} \approx 5$ days. Notably, the isomerism only causes a lack of reactivity, not premature drug release, so the adverse effect on the therapeutic window was not severe. The activator was administered in mice two days after the ADC, resulting in highly efficacious treatment. Impressively the two-component treatment displayed greater antitumour activity than a protease-cleavable conjugate using the same antibody fragment. This data suggests that enzyme-cleavable linkers may not always be well suited to extracellular release at the tumour and an exogenous trigger can be superior, despite the increased complexity.

3. Enzyme cleavable linkers

In the classical mechanism of action, ADCs are trafficked to the lysosome of a cell where high concentrations of unique

hydrolytic enzymes reside, thereby offering an opportunity for enzyme cleavable linkers to be selectively cleaved intracellularly. For selective payload release in the extracellular tumour area, enzymes that are upregulated in tumour microenvironments must be exploited.

3.1. Dipeptide-containing linkers

Dipeptide-containing linkers are present in the majority of ADCs that have reached clinical trials, including the approved brentuximab vedotin (Adcetris®) (Fig. 1C).[8] Dipeptidic linkers are often cited to be targeting cathepsin B, a cysteine protease that is relatively exclusive the lysosomal compartment in healthy mammals, with high expression across mammalian cells. Cathepsin B is also commonly implicated in tumour progression, with overexpression and extracellular activity observed in a wide range of cancers. Cleavable dipeptides were first developed as cathepsin B-cleavable linkers for Doxorubicin (Dox)-prodrugs in 1998.[29,30]

3.1.1. Initial development. Previously known lysosome-cleavable tetrapeptides Gly-Phe-Leu-Gly and Ala-Leu-Ala-Leu were unsuitable for prodrug or ADC applications due to their slow release kinetics, hydrophobicity and complexity. However, the carboxydipeptidase activity of cathepsin B enables it to cleave a dipeptidic linker with a payload attached at the C-terminus. The previously described Phe-Arg sequence, a general substrate for cathepsin B and L, was used as a starting point for investigations of dipeptide-cleavage rates by cathepsin B (Fig. 12).[29,30] Two general trends were observed: (1) a hydrophilic residue at P_1 is required and hydrolysis rates increase with basicity. Citrulline (Cit) is isoelectronic with Arg, and was preferred to Arg due to synthetic ease, despite the lower basicity. (2) The hydrophobic residues Phe, Val and Ala at P_2 enable cleavage by cathepsin B whilst imparting plasma stability.

Due to the steric bulk of the doxorubicin payload, a spacer unit was required for enzymatic activity. A *para*-aminobenzyl carbamate (PABC) linkage was used as a self-immolative spacer, which spontaneously undergoes a 1,6-elimination upon proteolysis to release Dox, CO_2 and aza-quinone methide (Fig. 13). Whilst Gly was also an effective spacer unit, the self-immolative nature of PABC is required to release the naked, unmodified payload. The spacer maintains enzymatic activity independent from the payload, increasing the applicability of this technology. All of these linker combinations were stable in isolated human plasma.

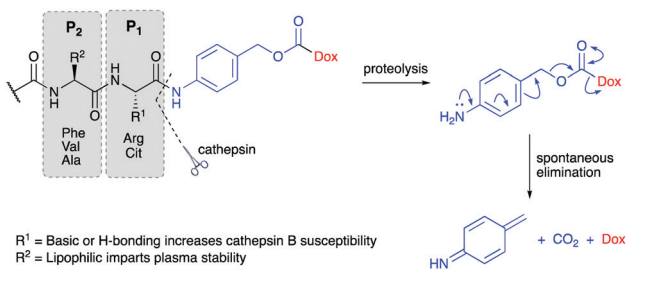

Fig. 13 The structure and cleavage mechanism of the Aaa-Aaa-PABC-Dox motif.

Dubowchik *et al.* subsequently applied their prodrug technology to ADCs with the Dox payload and an internalising BR96-antibody.[31] Despite the faster hydrolysis of Phe-Lys over Val-Cit in isolated cathepsin B, the hydrolysis rates were equal when the dipeptide linkers were incubated in rat liver lysosomal extracts. This suggests that other lysosomal enzymes contribute to the hydrolysis. As expected, upon conjugation of the dipeptidic linkers to the mAb, the hydrolysis rates by cathepsin B and rat liver lysosomes were greatly reduced, due to the steric bulk of the antibody hindering the enzymes. The potency of the conjugates were similar to unconjugated Dox in the BR96-overexpressing cells, but were >550-fold less potent against BR96-negative cells, signifying increased selectivity over unconjugated Dox. The dipeptide-containing ADCs were dramatically more selective and potent than an analogous acid-cleavable hydrazone-containing ADC, demonstrating the benefits of selective enzyme-mediated drug release.

Phe-Lys-PABC and Val-Cit-PABC containing linkers were also successfully applied to ADCs bearing the more potent MMAE payload.[13] When incubated in isolated human plasma, the dipeptide-containing ADCs exhibited long half-lives of 30 and 230 days respectively. In mouse plasma, however, the conjugates were less stable with $t_{1/2}$ = 12.5 and 80 hours for Phe-Lys and Val-Cit respectively. Whilst these dipeptidic linkers showed vastly improved human/mouse plasma stability over a hydrazone and ester-containing linker, their lack of long-term stability in mouse plasma, especially for Phe-Lys, could still lead to off-target toxicity in preclinical studies. Nevertheless, the Val-Cit-containing ADC was highly active *in vivo*, with a large therapeutic window in mice despite the mouse plasma instability.

3.1.2. Improving the mouse plasma stability of Val-Cit. Due to the importance of PK/PD studies in mice, the mouse plasma instability of Val-Cit may lead to failure in pre-clinical testing. Indeed, Dorywalska *et al.* demonstrated that the potency and activity of Val-Cit containing ADCs is reduced by this instability.[10] The premature drug release in mouse plasma was shown to be enzymatic and highly dependent on conjugation site, with more solvent-exposed sites exhibiting lower stability. Furthermore, the rate of hydrolysis *in vivo* was much higher than in isolated mice plasma, possibly due to a continuous supply of the enzyme. Surprisingly, the enzyme responsible for the instability was not a cathepsin, but instead showed sensitivity to a serine hydrolase inhibitor.

The nature of the enzyme responsible for the unwanted cleavage was revealed in 2016 when the same group used serum fractionation and proteomic analysis to identify it as carboxylesterase 1C (Ces1C).[32] Using Ces1C knockout mice, a Val-Cit-containing ADC was fully stable, confirming that Ces1C is the enzyme responsible for the instability of Val-Cit linkers in mice. The catalytic serine residue of Ces1C likely lies very deep inside a narrow pocket, in contrast to cathepsin B's shallow active site that makes it more promiscuous. Minor modifications to the Val-Cit linker were therefore expected to eliminate unwanted extracellular drug release whilst maintaining rapid hydrolysis by cathepsin B and other lysosomal enzymes. The addition of

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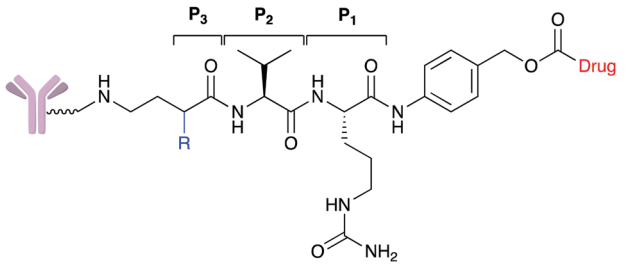
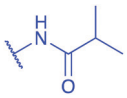
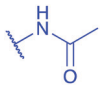
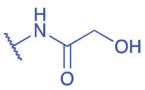
Table 1 The stability of modified Val-Cit-PABC-containing ADCs in rodent plasma. The shaded entry signifies the linker used in an ADC that outperformed the unmodified variant in a mouse xenograft model



R group	% Stability after 4.5 days	
	Mouse plasma	Rat plasma
	0	75
	5	94
	65	96
	84	97

hydrophilic P_3 residues at the N-terminus increased the mouse plasma stability of the ADC, without negatively affecting cathepsin B-cleavage (Table 1). When tested *in vivo*, an ADC bearing a more stable linker was significantly more efficacious against a mouse xenograft, inhibiting tumour growth for longer than in the unmodified Val-Cit linker. This study demonstrates the importance of stability in plasma for efficacious ADCs as well as specifically highlighting the importance of Val-Cit stability in mouse models.

More recently, Anami *et al.* investigated tripeptidic linkers with more hydrophilicity at P_3 to eliminate cleavage by Ces1C and provide improved aqueous solubility (Fig. 14).[33] Whilst P_3 = Ser gave only slightly increased stability and P_3 = Lys reduced stability over the native Val-Cit, the use of acidic Glu and Asp residues dramatically increased linker stability, with Glu slightly outperforming Asp (Fig. 14). Clearly, an acidic P_3 residue is effective at repelling the Ces1C enzyme. Furthermore, the Glu-Val-Cit-containing linker exhibited increased hydrolysis by cathepsin B. When ADCs bearing the linkers Val-Cit, Ser-Val-Cit and Glu-Val-Cit were incubated in mouse plasma, Glu-Val-Cit demonstrated full stability over 14 days whereas the others were almost fully hydrolysed over this time period. Whilst the effects of different linker stabilities were not observed *in vitro*, *in vivo* studies clearly showed superiority of a Glu-Val-Cit-containing ADC over the less stable linkers, curative at 3 mg kg^{-1}.

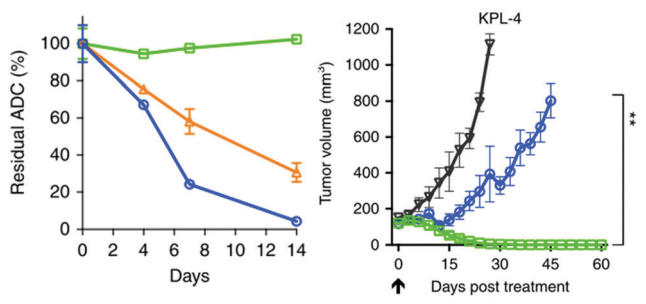


Fig. 14 The *in vitro* mouse plasma stability and *in vivo* antitumour activities of Glu-Val-Cit (green) and Val-Cit (blue) containing ADCs, stability data for the Ser-Val-Cit ADC (orange) is also included. Reproduced from ref. 33, used under license CC BY.

3.1.3. Understanding the role of cathepsin B. Although cathepsin B was initially targeted as the primary enzyme for cleaving dipeptidic Val-Cit linkers, a growing body of evidence has refuted this. The lack of specificity towards cathepsin B was best exemplified in 2017 when workers at Genentech and 23andme revealed cathepsin S to be the most active enzyme in the family towards a Val-Cit-containing ADC, in isolated enzyme incubation studies.[34] The researchers then suppressed cathepsin B expression in cells using CRISPR-Cas9 gene deletion and shRNA knockdown. When Val-Cit-containing ADCs were tested in these cells and in cathepsin B-expressing cells, no difference in efficiency of MMAE release or potency was observed. Despite a lack of *in vivo* confirmation, this data clearly points to the redundancy of cathepsin B in the role of ADC payload release, despite it being the original target for enzymolysis.

In another study, chemists at Genentech and Spirogen used a classical medicinal chemistry approach to specifically target cathepsin B.[35] Cathepsin B has been more widely implicated in cancer cell metastasis than other lysosomal proteases and its role in degradation in the extracellular matrix is beginning to be more deeply understood. An ADC whose linker is more specifically dependent on cathepsin B may therefore be more efficiently released both intra- and extracellularly in tumours, thereby increasing the therapeutic window. The researchers sought to remove the P_1–P_2 amide bond whilst maintaining some H-bond interactions, as well as replacing the P_2 residue, eventually settling with the cyclobutane-1,1-dicarboxamide (cBu) moiety (Fig. 15). Indeed, the new peptidomimetic structure did form these interactions and its V_{max}/K_m with cathepsin B was only 4.5-fold less than the Val-Ala-containing model linker. cBu-containing ADCs carrying MMAE or PBD-dimer payloads exhibited equal or better efficacy against mouse xenografts. Intracellular-cleavage of the cBu-Cit-containing ADC was inhibited 75% by a cathepsin B-specific inhibitor and 90% by

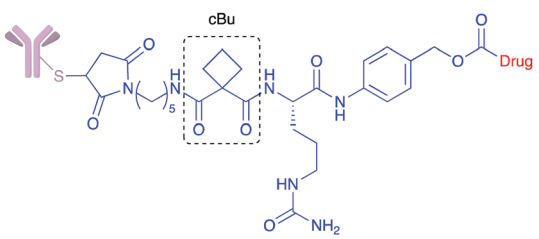


Fig. 15 The structure of the cBu-Cit-PABC-containing ADCs where drug = MMAE or PBD-dimer.

a broad-spectrum protease inhibitor. However, only the broad-spectrum inhibitor could inhibit the Val-Cit-containing ADC. The cBu-Cit moiety is therefore more selective towards cathepsin B than Val-Cit and does not require other proteases in order to effectively release the payload.

3.1.4. The Val-Ala motif. Val-Cit-containing linkers are the most studied and clinically developed dipeptides, but the Val-Ala moiety has also emerged as an effective protease-cleavable group, with numerous examples in clinical trials. Like Val-Cit, Val-Ala is effectively cleaved in cells and it is highly stable in human plasma. The Val-Ala unit was first applied to ADCs in 2006 with highly potent Dox derivatives as payloads.[36] In an isolated cathepsin B-cleavage assay, the Val-Ala linker was cleaved at half the rate of the Val-Cit linker, but it exhibited lower hydrophobicity. The importance of this was exemplified in the conjugation of the ADCs, where in the case of Val-Ala, 7.4 drugs per mAb were loaded without significant aggregation (>10%) occurring. Contrastingly, the Val-Cit-containing linker could not reach a DAR > 4 because of excessive aggregation and precipitation.

This propensity to create less hydrophobic, more soluble linker-payload constructs is at the centre of Val-Ala's success. Recently, a surge in interest in highly lipophilic, PBD-dimer payloads has boosted the popularity of Val-Ala-containing linkers. One example is rovalpituzumab tesirine, currently in Phase II and III trials for small-cell lung cancer (Fig. 16).[37] In conjugates bearing MMAE payloads, the ADC aggregation of the Val-Ala-containing linker was lower than for the Val-Cit analogue whilst the *in vitro* and *in vivo* tests showed no observable difference in potency, efficacy or toxicity.[38]

Although no side-by-side comparison of the mouse plasma stability of Val-Ala and Val-Cit linkers has been conducted in ADCs, such a study was conducted with small molecule drug conjugates in isolated mouse serum and the half-lives for Val-Ala and Val-Cit were 23 h and 11.2 h respectively.[39] This result suggests that although slightly improved, Val-Ala-containing ADCs will likely exhibit the same instability problems in mice as Val-Cit unless the conjugation site is enzyme-inaccessible, as in the case of rovalpituzumab tesirine.

3.1.5. Non-internalising dipeptide-ADCs. The vast majority of research in the field of dipeptide-containing cleavable linkers has focused on the traditional ADC mechanism of action whereby internalisation is followed by enzymatic cleavage in the lysosome. However, lysosomal enzymes are known to be overexpressed extracellularly in a wide range of cancers.

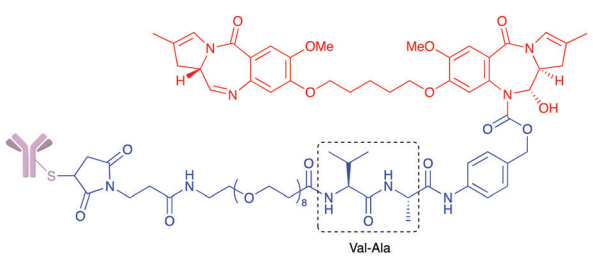


Fig. 16 The structure of rovalpituzumab tesirine.

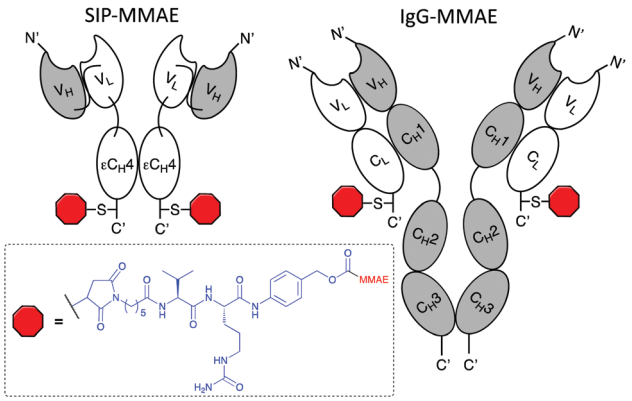


Fig. 17 The structures of the non-internalising SIP- and IgG-ADCs.

This has been exploited in albumin–drug conjugates, polymer–drug conjugates and prodrugs, where endocytosis is not usually necessary to effect cytotoxicity.[40] In these cases, extracellular cleavage at the tumour site allows selective drug delivery. As with the reducible disulfides, dipeptide-linkers are likely to benefit from a chain-reaction, whereby the initial death of a tumour cell will release lysosomal enzymes into the extracellular area, further triggering linker cleavage and payload release.

In 2017, having already developed disulfide-containing non-internalising ADCs, researchers in the Neri laboratory used non-internalising ADCs bearing Val-Cit-PABC linkers to target the tumour extracellular space.[41] As before, the antibodies were expressed in both IgG and SIP format and the ADCs were compared (Fig. 17). The ADC utilising the SIP antibody was unstable in isolated mouse serum, presumably hydrolysed by the Ces1C enzyme. However, in the larger, more hindered IgG format, the ADC was completely stable in isolated mouse plasma, but not *in vivo*. The enzymatic source of payload release *in vivo* has not been determined, but cleavage at the tumour microenvironment appears to be effective. Impressively, the IgG ADC was efficacious *in vivo* against two different xenograft models, curative at 7–10 mg kg^{-1}. The preference of this system towards the IgG antibody format contrasts with the ADCs bearing reducible disulfides, previously discussed.[25]

Continuing their research, workers in the Neri laboratory further explored the scope of dipeptides suitable for the application of extracellular release in tumours.[42] The antitumour activities of a range of ADCs bearing dipeptide linkers in the form Val-P_1 where P_1 = Arg, Lys, Cit and Ala were assessed in mice. Up to dosage levels of 50 mg kg^{-1}, all the ADCs were well tolerated. When xenografted mice were treated with 3 mg kg^{-1} of the ADCs, improvement was observed in all cases over the saline control, with Val-Arg exhibiting the worst tumour growth inhibition and Val-Ala the best. The stability and metabolic fate of each of the ADCs was compared and whilst Val-Cit and Val-Ala displayed the predicted cleavage at the P_1 C-terminus, the ADCs bearing a basic P_1 residue (Lys or Arg) were cleaved at the amide between P_1 and P_2, therefore unfunctionalised MMAE may not have been released. This unwanted hydrolysis site made these more basic residues unsuitable for further

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development in this study. Crucially, an ADC containing a non-cleavable linker was inactive in a tumour model in which Val-Ala and Val-Cit both showed full tumour regression. Although a promising technology, extracellular enzyme-cleavable ADC linkers are as yet unproven in primates, where enzyme distribution varies from rodents.

3.2. Glycosidase-cleavable linkers

3.2.1. β-Glucuronidase-cleavable linkers. β-Glucuronidases are hydrolytic lysosomal enzymes in the glycosidase class that catalyse the breakdown of β-glucuronic acid residues in polysaccharides. Their exclusivity to the lysosomal compartment and the hydrophilicity of their β-glucuronic acid substrates make them ideal candidates for enzyme-cleavable linkers for ADCs. Similarly to cathepsins, β-glucuronidases are secreted in some tumours to necrotic areas, where they can function extracellularly. This extracellular activity led to the development of β-glucuronidase-cleavable prodrugs as early as 1988, whereby selectivity for the tumour was achieved by extracellular enzymatic cleavage in the vicinity of malignant cells.[40] Interest in β-glucuronidase-cleavable prodrugs has been extensively studied since, with applications reaching to antibody-directed enzyme prodrug therapy (ADEPT) and albumin–drug conjugates.

However it was not until 2006 that β-glucuronidase-cleavable linkers were applied to ADCs.[43] This preliminary research from Seattle Genetics employed a β-glucuronic acid unit attached to a PABC spacer as seen previously with dipeptidic linkers. In this case, attachment to the antibody was achieved by substitution of an amide bond *ortho*- to the enzyme-cleavable group (Fig. 18). The resulting ADCs employed MMAE, MMAF and doxorubicin derivatives as payloads and antibody conjugation was achieved with low levels of aggregation, even with drug loading as high as 8.3 drugs per mAb. Crucially, the conjugates were highly stable in isolated rat plasma, with an extrapolated half-life of 81 days, far superior to the dipeptidic linkers. The linkers efficiently released the payloads upon incubation with β-glucuronidase and after *in vitro* testing, analysis of cell extracts showed release of unmodified payload in antigen positive cells, although the release was not quantified. As expected, the conjugates were potent *in vitro* and highly active *in vivo* in mice. Interestingly, the maximum-tolerated dose of the β-glucuronic acid ADCs was similar to the analogous dipeptide ADCs, despite the inferior plasma stability of dipeptides in mice.

Since their first publication on the topic, Seattle Genetics have employed their β-glucuronic acid motif with a wide range of lipophilic payloads to create non-aggregated ADCs. When employing camptothecin analogues as payloads, ADCs bearing hydrophilic β-glucuronic-acid linkers displayed <5% aggregation at DAR = 8, compared to the Val-Cit-containing ADCs, which were >80% aggregated.[44] This huge increase in monomeric ADC formation in comparison with Val-Cit-conjugates is consistently observed. When comparing DAR = 4 ADCs, the β-glucuronidase-cleavable ADCs were more efficacious than Val-Cit ADCs in xenografted mice. Curiously, the maximum-tolerated dose (MTD) of the Val-Cit ADC was higher than the β-glucuronic acid linker, for reasons that remain unclear. In another direct comparison between Val-Cit and β-glucuronic acid-containing ADCs, the effects of bystander killing were investigated through the use of admixed tumour xenografts, containing both antigen positive/negative cancer cells.[45] In this *in vivo* model, the β-glucuronic acid MMAE ADC and the Val-Cit-MMAE linker were equally active at reducing the overall tumour volume.

Whilst utilising their existing β-glucuronidase-cleavable technology, the researchers at Seattle Genetics developed a novel linker construct capable of releasing alkyl alcohol-linked payloads.[46] A methylene-alkoxy appendage was added to the PAB spacer to allow the release of alcohols, which are unable to spontaneously eliminate from PAB-spacers, due to their insufficient leaving group ability (Fig. 19). Although some optimisation of the construct was required, the eventual structure containing an *N*,*N*-dimethyl ethyl-group was stable at pH 7.4 and efficiently eliminated the payload upon enzyme hydrolysis.

The emergence of the hydrophilic β-glucuronic acid has enabled the synthesis of highly loaded ADCs with DAR = 8 without forming highly aggregated ADCs. Alongside the reduction in aggregation, reducing hydrophobicity in highly loaded ADCs greatly reduces plasma clearance, thereby increasing exposure and *in vivo* efficacy.[47] For hydrophobic Val-Cit-containing ADCs, activity is greater at DAR 4 than DAR 8 due to this increased clearance. However, when hydrophilic β-glucuronic acid linkers were employed, DAR 8 ADCs became achievable without compromising pharmacokinetics. In addition to these findings, further increasing hydrophilicity of β-glucuronic acid-based linkers with branched PEG chains greatly increased the ADC exposure and activity.

3.2.2. β-Galactosidase-cleavable linkers. More recently a β-galactosidase-cleavable linker has been described (Fig. 20).[48] Similarly to β-glucuronidase, this lysosomal enzyme is overexpressed in certain tumour types. The enzyme is analogous to β-glucuronidase in its hydrolytic activity, but instead hydrolyses β-galactoside. Impressively, the authors found that this type of

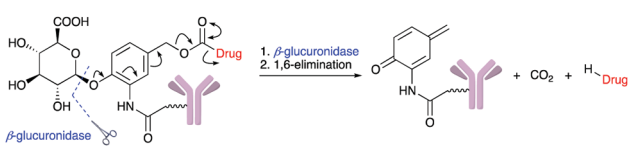

Fig. 18 The structure and release mechanism of β-glucuronic acid-containing ADCs.

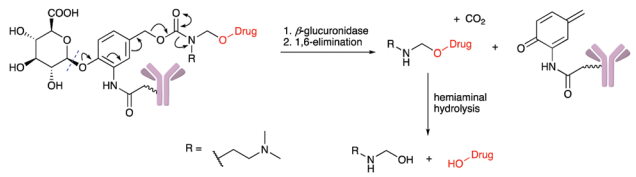

Fig. 19 The structure and release mechanism of a methylene-alkoxy-containing β-glucuronidase-cleavable ADC.

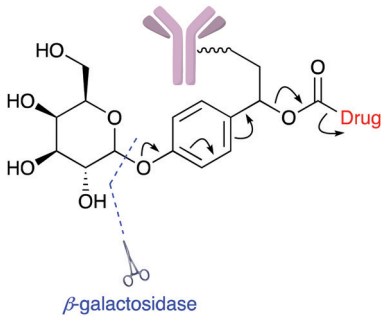

Fig. 20 The structure and release mechanism of an β-galactoside-containing ADC.

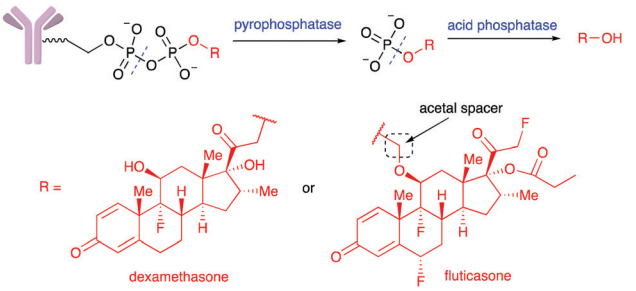

Fig. 22 The structure of cleavage mechanism of pyrophosphate-containing ADCs bearing glucocorticoid payloads.

linker, when used with trastuzumab and MMAE, was more potent than the Val-Cit-PABC analogue. Furthermore, this linker-payload combination was more efficient than the approved drug trastuzumab emtansine (T-DM1) for the treatment of HER2+ mammary tumours in mice.

3.3. Phosphatase-cleavable linkers

Lysosomal acid pyrophosphatase and acid phosphatase are enzymes that hydrolyse pyrophosphates and terminal monophosphates respectively to their parent alcohols in the lysosome. Targeting these enzymes for drug release offers two distinct opportunities: (1) the substrates are naturally highly hydrophilic, and (2) alkyl alcohol payloads can be released.

In 2016, researchers at Merck employed a terminal phosphate/pyrophosphate as a leaving group for the known Val-Cit-PAB cleavable linker system.[49] Upon lysosomal proteolysis of the Val-Cit group, the PAB unit was able to eliminate a terminal monophosphate (Fig. 21). The terminal phosphate was hydrolysed by acid phosphatase upon elimination, releasing an alcohol payload in an overall dual-enzyme process. When incubated in lysosomal extracts, efficient payload release was observed, with minor accumulation of the monophosphate intermediate. Although pyrophosphatase and acid phosphatase are highly likely to be the enzymes responsible, this putative mechanism of action is unproven. The hydrophilic linkers facilitated the conjugation of budesonide, a lipophilic glucocorticoid. The ADC bearing the linker was active and selective *in vitro* but there has of yet been no *in vivo* validation of this approach. Furthermore, plasma stability studies were limited to only six hours, insufficient for the lifetimes associated with ADCs.

More recently Merck have also demonstrated novel, highly simplistic pyrophosphate-containing linker for the release of alcohol-linked payloads.[50] In their design, a phosphate diester

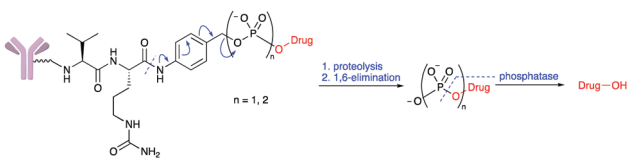

Fig. 21 The structure of cleavage mechanism of a dipeptide-phosphate-containing ADC linker.

is incorporated into an alkyl chain. The linkers were hydrolysed in lysosomal extract media, first releasing the monophosphate-appendaged alcohol payload, then the unfunctionalised glucocorticoid drug (Fig. 22). When a linker containing only a monophosphate was tested in lysosomal extracts, drug release was unsuitably slow. The phosphate diester strategy was effective for an unhindered dexamethasone payload but with the bulkier fluticasone propionate, enzymatic release was sluggish. The researchers mitigated this slow drug release by introducing an acetal spacer and the resulting linker-drug showed similar hydrolysis rates to the dexamethasone–pyrophosphate linker in lysosomal extracts. ADCs bearing the two linker-payload combinations were tested for stability in mouse and human plasma and full stability was observed over seven days. It is likely that an initial pyrophosphatase enzyme mediates phosphate diester hydrolysis and a second acid phosphatase enzyme cleaves the terminal monophosphate to reveal the unmodified payload. Tested *in vitro*, the two ADCs bearing pyrophosphate-linked payloads were highly potent, unlike ADCs containing only a monophosphate. It was therefore suggested that potency is highly dependent on payload release rate in this system. The pyrophosphate-containing drug-linkers were soluble, above 5 mg mL^{-1}, allowing conjugation of these lipophilic glucocorticoids in aqueous media.

4. Conclusions and future outlook

Suitable linker technology at the payload attachment site is a proven requirement for safe and efficacious ADCs. Despite the few examples of effective non-cleavable linkers, cleavable linkers are the preferred choice in the treatment of the majority of cancer types due to the importance of bystander killing and payload cytotoxicity. Acid cleavable ADC linkers were initially promising but the stringent stability requirements imparted by highly potent payloads has reduced their value. Contrastingly, the vast majority of current ADCs employ either disulfide or dipeptide technologies due to their ability to effectively differentiate between plasma and target cell conditions. Both these technologies are now well established and are being further developed to better address the key issues of solubility, plasma stability and the ability to release a number of functional groups. Furthermore, they have demonstrated applicability towards non-internalising ADCs by extracellular cleavage.

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Access to this alternate mechanism of action vastly increases the number of possible antigen targets by removing the requirement for antigen internalisation, which may open the door to many new ADC therapies. Future research of cleavable linkers is expected to further explore the possibility of this alternative mechanism of action, including the area of exogenous stimuli, which is in its infancy.

Non-dipeptide based enzyme-cleavable linkers have not yet been clinically validated, despite their encouraging preclinical results. Glycosidase- and phosphatase-cleavable linkers have exhibited excellent solubility and stability profiles and therefore should be expected to have a role to play in the future clinical landscape of ADCs.

Conflicts of interest

There are no conflicts to declare.

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