university of groningen / faculty of science and engineering

Essay

Bispecific Antibody-Drug Conjugates

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Abstract

Bispecific antibodies have gain great interest the last decades and relevant studies have come up with new possibilities in their therapeutic applications. A special interest is acquired in the design of bispecific antibody drug conjugates, that allows targeted delivery of therapeutic payloads in the body, with great specificity and efficacy. Many technologies have been developed for the successful design of bispecific ADC, focusing on the production of different bispecific ADC formats, the optimization of their efficacy, their linkage with the drug and the way of their administration. This essay will review these aspects as well as the technologies designed on them and it will present relevant studies.

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1.Introduction

1.1 Immunity

Advanced vertebrate animals, including humans, are empowered with immunity to protect against foreign and endogenous harmful substances, called antigens, while distinguishing them from harmless endogenous and exogenous substances. The immune system includes physical, chemical and cellular barriers that either prevent the spread and movement of foreign pathogens, malignant cells and harmful substances (innate immunity) or combat against them when they have invaded into the body (both innate and adaptive immunity). Adaptive immunity manifests high molecular specificity for its target antigen and is distinguished in cellular and humoral immunity which are mediated by antigen specific T-cells and circulating antibodies, respectively[1]. Antibodies are glycoproteins that are produced by plasma B cells and recognize and bind antigens with high affinity and great specificity[2].

1.2 Antibody structure

The chemical structure of antibody molecules, also called immunoglobulins, was identified in the 1970s by scientists Edelman and Porter, who shared the Nobel Prize in Physiology and Medicine for this discovery in 1972[3]. Immunoglobulins have a Y shape and they are composed of two antigen-binding domains (Fabs) bound to the fragment crystallizable (Fc) by a hinge region domain. Each oligomer consists of 4 polypeptide chains, 2 identical heavy chains (H) and 2 identical light chains (L) that are linked together by disulfide bonds. In each chain, there is one variable domain (LV, HV), which sequence varies among antibodies, and constant domains (LC, HC), which are similar for immunoglobulins of the same antibody group or class and determine their function. The antibodies are classified into five main isotypes, the IgA, IgD, IgE, IgG and IgM (figure 1), with IgG having the subclasses IgG1, IgG2, IgG3 and IgG4 and being the most abundant antibody in normal serum. The variable domains LV and HV are paired together, via disulfide bonds, at the amino terminal ends of the antibody to form the antigen binding sites, thus creating two paratopes (=antigen binding regions) for the same antigen, while the tail region of the antibody at the carboxyl termini is formed by HC chain pairing, via disulfide bonds, and it mediates biological activity[1,4,5]. For IgA and IgM, antibodies of a higher oligomeric complex comprising (Figure 1).

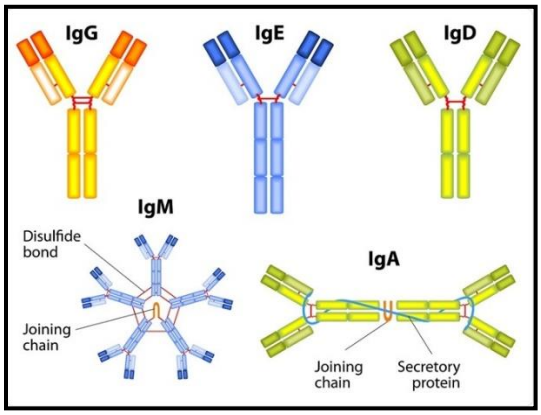

Figure 1: Representation of the 5 main antibody classes - IgG, IgE, IgD, IgM and IgA. (by Designua / Shutterstock)

1.3 Antibodies as therapeutic molecules

Structural knowledge of antibodies and an understanding of their function enable the engineering and development of such proteins for therapeutic applications. Their high specificity and affinity and their broad range of possible targets makes them in principle efficient medical tools against a wide range of diseases, while they are well tolerated because antibodies are natively present in the body[5]. In 1986, the first murine monoclonal antibody (mAb) was approved for therapeutic use, Muromonab targeting the T-cell protein CD3, used for anti-rejection treatment in patients with organ transplants[6]. Nevertheless, murine antibodies have properties that limit their application, such as creating an immunogenic response in the human body, which is basically another antibody response against the therapeutic antibody, and is triggered because of sequence differences between mouse and human antibodies. To minimize such immunogenicity, first chimeric and then humanized antibodies have been constructed. Chimeric antibodies are composed of human constant and mouse antigen-binding domains[7], while in humanized antibodies both domains are mostly human (except the paratopes), which makes them less immunogenic and more efficient concerning the interaction with human effector cells.[8]. Recently, CRISPR technology was used in antibody engineering to produce chimeric antibodies with the preferred Fc format, species and isotype and offering the possibility to fuse the antibody with tags or mutations without affecting antigen specificity[9,10]. The addition of tags like sortag enables the chemoenzymatic attachment of molecules that act as drugs against a specific target. A sortag is used in a sortase-mediated ligation operated by the bacterial transpeptidase sortase A, which catalyzes a ligation reaction between the sequence LPXTG and oligoglycine nucleophiles[11]. This technique accelerates the development of targeted drug delivery mediated by efficient optimized bispecific antibodies[10].

By now, up to 79 mAbs are used in clinic for therapeutic approaches, confirming their success as therapeutic tools, and approximately 3,700 are under development in clinical trials[12]. They can act in different ways, such as by blocking a signaling pathway by binding to a surface receptor after competing with a signaling ligand, recruiting immune-effector cells that bind their Fc domain, activate complement-dependent cytotoxicity (CDC) that forms a pore and lyse the cell, or deliver conjugated drugs to the target cell, like radioisotopes or toxins[13]. Nevertheless, their monospecificity limits their applicability to single targets. Via molecular engineering techniques, scientists managed to combine two different antibody-binding sites and create one single antibody molecule with two different paratopes: the bispecific antibody (bsAb), which hence recognizes two different epitopes. Interestingly, bispecific antibodies have been also found in vivo, formed by the exchange of the fab domains between two IgG4 antibodies in human and IgG1 antibodies in mouse, that recognize two different antigens[14]. The design of such molecules can advance the technology of therapeutic antibodies by enhancing selectivity towards the target, as the target cell can now be recognized by two markers instead of one, and providing new mechanisms of action, such as linking immune cells to the target cell, thus improving efficacy and safety. By now, three bispecific antibodies have been approved for therapeutic use and around 34 are currently tested in clinical trials[15,16,17,18]. The main mechanisms of action of these bispecific therapeutic antibodies are the recruitment of immune cells to the target, signaling blocking and drug delivery.

Many years before the first therapeutic antibody was developed, the concept was proposed, by the German physician Paul Ehrlich, of drugs identifying their target and selectively attack pathogens yet remain harmless within healthy tissues, with the designation ‘magic bullet’. This idea has inspired the development of target-selective drugs and led to the design of antibody-drug conjugates (ADCs)[19]. ADCs consist of mAbs connected covalently via a linker to therapeutic molecules that either directly kill the

cell, such as cytotoxic agents, like protein toxins, small molecules that inhibit or modify DNA, RNA or tubulin, isotopes like Iodine-131 that can be used in chemotherapeutics, or cytokines which stimulate antitumor immune response[20,21]. Combining the technologies of ADCs and bsAbs can benefit greatly the targeted delivery of payloads in sense of selectivity, efficacy and safety[18,22].

This essay will be focused on bispecific antibodies and their application as payload delivery molecules. The different formats of bsAb design will be reviewed and their construction as ADC molecules will be analyzed. The ways of enriching the main prerequisites for ADC formation, selectivity and efficacy, will be introduced and how these molecules are administrated into the body and used in clinical trials will be presented.

2. Bispecific formats

Bispecific antibodies have two different antigen-binding domains (Fab). Two major classes of bsAbs can be distinguished, those lacking an Fc region, so called single-chain variable fragment (scFv)-based antibodies (figure 3A), and those bearing an Fc region, the full-length IgG-based antibodies (figure 3B). There are different advantages and disadvantages in the absence and presence of Fc fragment depending on the application. For example, scFv-based antibodies have simpler design and smaller size which enables them to penetrate better in tissues. Nevertheless, due to their small dimensions they have a shorter half-life in vivo because of rapid renal elimination. On the contrary, the presence of Fc fragment results in a longer half-life, because of its larger size and the FcRn-mediated recycling, better solubility and stability, easier purification and other Fc-mediated effector functions, such as antibody-dependent cytotoxicity (ADC), a mechanism that activates cellular immune response against antibody-cell complex, complement-dependent cytotoxicity (CDC), which attracts and activates pore forming proteins that lyse the cell, and antibody-dependent cellular phagocytosis (ADCP), that activates macrophages to induce phagocytosis [23],[24],[25].

2.1 scFv-based bispecific antibody formats

Even though Fc-less bsAbs appear to have a simple structure and mainly consist of VL and VH domains, many different variations have been designed, including but not limited to tandem scFVs, diabodies, dual-affinity re-targeting proteins (DARTs) and tandem diabodies (TandAbs) (figure 3A). First, tandem scFv molecules are composed of two different scFvs, each of them forming a single Fab, fused by a flexible linker. This is the format of bispecific T-cell engager (BiTE) molecules, which target T-cells to tumor cells, by binding the CD3 T-cell receptor and the tumor antigen, respectively[25]. Second, diabodies are constructed differently, because now the fragments comprising VH and VL domains are connected by a linker that is too short to allow pairing between them. Instead, the fragments are then forced to pair with other complementary fragments to create two antigen-binding sites[26]. To induce correct pairing and stability, an interdomain disulfide bond can be introduced in one of the VH-VL pairs, creating the DARTs[27]. To increase the residence time of such small molecules in the body and increase their stability, TandAbs were generated by adding more scFvs at the polypeptide chains, creating tetravalent molecules that contain two binding sites for each antigen and have almost the double size of a simple diabody, offering a longer half-life[28].

2.2 Bispecific IgG molecules

Bispecific IgG antibodies have some similar properties as the natural IgG immunoglobulins, like the Y shape, but they distinguish from them in their asymmetric architecture that is generated by the presence of at least two different Fv regions. They are composed of two different heavy and two different light chains that form an Fc and two distinct Fab fragments which recognize different epitopes. The correct assembly of these chains may be challenging as there are 16 combinations possible from which only two form the desired fragments, whereas the other 14 form either non-functional or monospecific molecules (figure 2). To minimize the heavy-heavy and heavy-light mispairing many technologies have been developed, which are presented below[25].

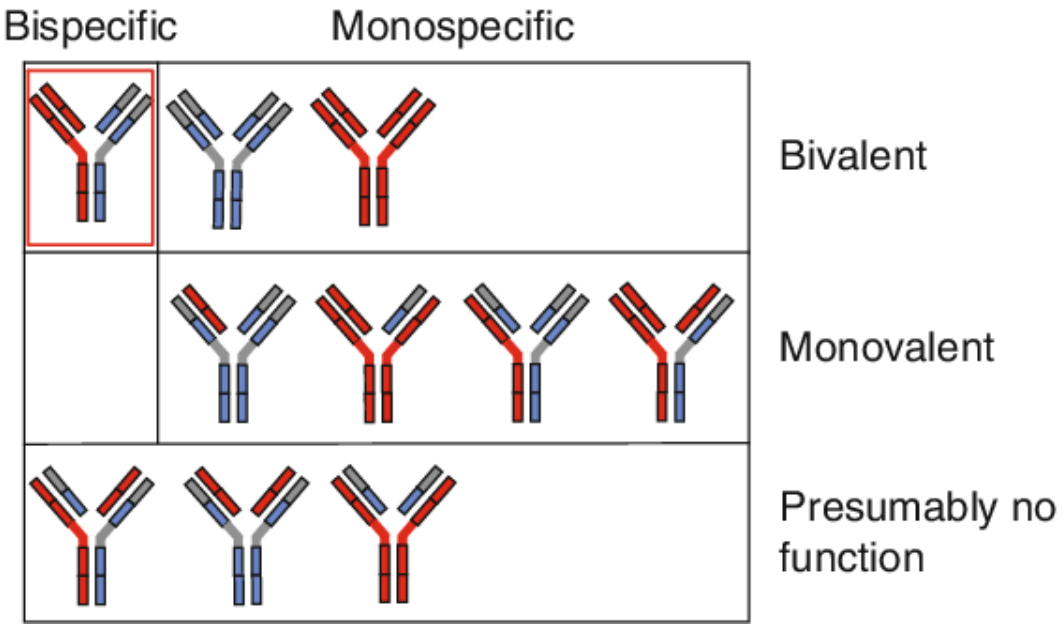

Figure 2: Chain association combinations in bispecific IgG antibody production. In total 16 formats are possible. Six of the formats, including the desired bispecific antibody, occur twice and the other four occur once[29].

2.2.1 H-H chain pairing

As the dimerization of heavy chains is achieved by the C-terminal heavy constant domains (CH3), the technologies developed to minimize the heavy-heavy mispairing focused on engineering these domains. Some examples are the Knobs-into-holes (KiH) technology[30], electrostatic steering effects[31], strand-exchange engineering domain (SEED)[32] and X_mAb bispecific platform[33] (figure 3B). The KiH technology introduced mutations to create a 'knob' in the CH3 domain of the first heavy chain, that was represented by a large amino acid and it was designed to insert into a 'hole' in the CH3 domain of the second heavy chain that was created by a small amino acid. Mutations that generate cysteine residues at specific positions located at the interacting surface and favor disulfide bonds further enhance correct pairing[30]. SEED is one more mutation-based technique which combine structurally related sequences of immunoglobulin A and immunoglobulin B, generating two asymmetric but complementary CH3 domains

in order to promote heterodimerization[32]. Another approach was the creation of electrostatic steering effects between the two CH3 domains inserting negative charged amino acids in the first chain and positively charged amino acids in the second chain. Attractive interactions favored while repulsive charge interactions suppressed heterodimerization, leading mostly to the creation of the desired Fc domain[31]. Finally, the X_mAb bispecific platform does not induce correct heterodimerization but proposes ion exchange chromatography as purification method for proteins containing a heterodimeric Fc domain taking advantage of their isoelectric point differences[33].

2.2.2 H-L chain pairing

The technologies mentioned cause a two-fold increase in the yield of correct IgG-like bispecific proteins as they favor the correct H-H assembly. Nevertheless, non-desired products are still formed due to H-L mispairing. Scientists have developed techniques, like common light-chain strategy and CrossMab (figure 3B), to minimize these mispairings and they can be used in combination with the previous mentioned technologies to efficiently produce correct whole IgG-like bispecific antibodies. First, the common light-chain strategy is based on the limited repertoire of light chains in a phase display antibody library, where common light chains can be identified in antibodies that recognize different antigens. Using the same light chain in a bispecific format simplifies the design and maximizes the correct yield, regarding the correct H-L pairing. Nevertheless, this technique limits the freedom in antibody engineering, and for instance limits the possibility to improve other properties, like pI modification and pH dependency, that optimize the resulting product[34]. Second, the CrossMab technology, relies on a crossover between the domains of the light and heavy chains in the Fab region of the bispecific antibody, to enforce the correct pairing. The way this works is that different domains within the Fab-fragment can be exchanged, thus the light domains become part of the heavy chains and vice versa, resulting in 3 main formats. In the $Cross^{Fab}$ format, the entire Fab domain is exchanged, in the $Cross^{VH\text{-}VL}$ only the variable domains of the Fab are exchanged, and last in the $Cross^{CH1\text{-}CL}$ format only the constant domains of the Fab are exchanged (figure 4)[35].

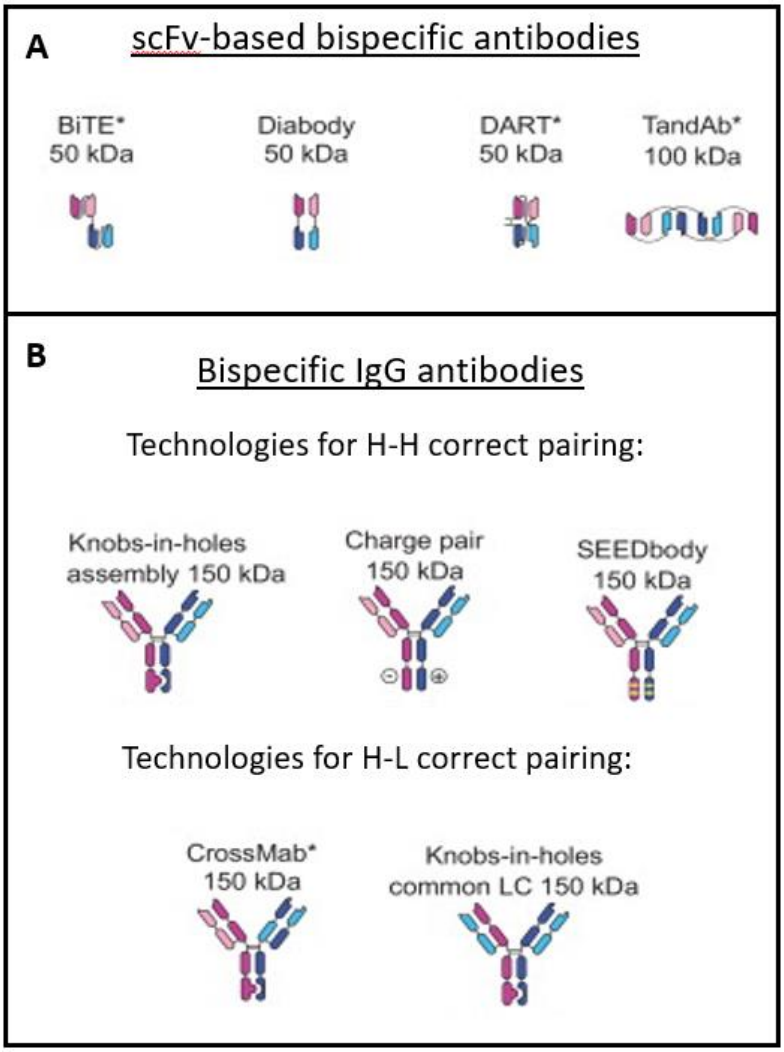

Figure 3: A. scFv-based bispecific antibody formats: BiTE, Diabody, DART, TandAb. B. Bispecific IgG antibody formats optimized by H-L correct pairing technologies: Knobs-into-holes, Charge pair (Electrostatic steering effects), SEED technology and L-L correct pairing technologies: CrossMab and Common light chain technologies combined with Knobs-into-holes technology[36].

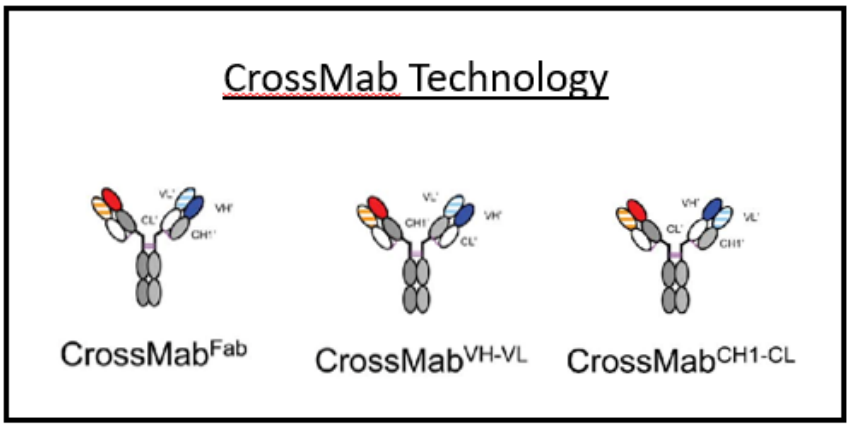

Figure 4: CrossMab technology formats: Cross^{Fab}, Cross^{VH-VL}, Cross^{CH1-CL} [37].

3. Bispecific antibody-drug conjugate

A bispecific antibody-drug conjugate (bsADC) needs to fulfill some requirements to be successful in therapy, mainly sufficient high affinity, specific binding and controlled activity. First, they need to recognize and act only at the target cells while leaving the healthy cells of the body unaffected. Bispecific antibodies can be more target-specific than monospecific antibodies as they can be designed to recognize a combination of epitopes. As there are single epitopes for each target antigen, the affinity is not very high eliminating cytotoxicity towards off-target cells in case of antigen expression in both healthy and target cells. Furthermore, the antigens chosen should be expressed only or mostly in the target cells and displayed on the surface so that they are accessible by the antibody. When administrated in the body, the bsADC should not be active on its way to the target but only in the microenvironment of the cell after surface binding or inside the cell after internalization. This can be controlled by the linker used to capture the drug and inactivate it temporarily. In this chapter we will discuss in more detail the mechanism of action, ways to optimize bsADC's internalization and thus efficacy, the linker design and the ways of administration[22].

3.1 BsADC mechanism of action

Cells internalize membrane proteins via endocytosis pathways that most often lead to lysosomal compartments. Such proteins can be membrane receptors that form complexes as soon as they bind their ligands and can then be internalized by endocytosis[38]. ADCs, like other antibodies that are bound to internalizing ligands on the cell membrane can end up in lysosomes. When ADCs internalize and reach the endosome-lysosome pathway, they can release the drugs either by linker cleavage or by proteolytic degradation of the antibody due to acidic conditions and action of lysosomal protease. There are two main internalization routes, the clathrin-mediated and the caveolae-mediated endocytosis, which are antigen-dependent[39],[20].

The canonical route for internalization of antibodies and ADCs is clathrin-mediated endocytosis. Clathrin is one of the cytosolic proteins that is the main component of the so-called clathrin coat. First, clathrin proteins assemble on the plasma membrane and bind to the lipids and to the cargo proteins, forming a

clathrin-coat. This coat creates a pit that with the association of polymerized actin filaments and scission modular proteins, such as endophilins and amphiphysins, is sealed and become clathrin-coated vesicles. The endocytic machinery is disassembled in the cytosol by uncoated-modular proteins and the vesicle is fused with endosomal compartments and lysosomes[39],[40].

Caveolae-mediated endocytosis is another internalizing mechanism that can occur in cells, but it is still controversial if the cellular fate of internalized caveolae differs from clathrin-mediated endosomes and these compartments traffic to distinct cellular compartments. Caveolae are cave-like membrane structures that are formed by the oligomerization of caveolin proteins in the membrane and their coating by some other proteins named p-cavin. The intracellular route of caveolae was revealed by the study of SV40 virus which was detected in multi-caveolar complexes of neutral pH, that did not fuse with lysosomes[41]. Two antibody-drug conjugates have been reported to be internalized via this endocytosis pathway. The first conjugate is an anti-CD20-auristatin molecule, which is composed of a synthetic antimitotic agent (auristatin), linked to an antibody targeting for the CD20 B-lymphoma cell surface antigen and inhibits the polymerization of tubulin in the target cells, preventing the formation of the mitotic apparatus. The trafficking of this antibody in human B-cell lymphoma cells was detected by fluorescence microscopy and it showed its internalization with both clathrin- and caveolae-mediated endocytosis[42]. The second one is an auristatin-containing antibody drug with affinity for melanotransferrin/p97. The melanoma cells with high surface p97 expression were sensitive to the drug, whereas the cells with low expression were resistant. Immunofluorescence microscopy revealed that the drug was still internalized in resistant cells via caveolae-mediated endocytosis but the absence of proteolytic activity in caveolae complexes did not lead to drug release[43].

In addition to internalization, it has been reported that ADCs can be effective when they bind non-internalizing receptors on the target cell. In this non-internalizing mechanism, the antibodies accumulate and release the drugs in the extracellular space of the target cells, causing a localized accumulation and damage. This enables cytotoxicity not only in cells that are antigen-positive but also in antigen-negative cells that form cellular malignancies. For example, the released drug can be internalized both in the tumor cells that are positive for the target and in the tumor endothelial cells that are negative for the target and are part of the tumor's environment. For the drug to release, it is important that the linker between antibody and drug is cleavable outside the cell, for instance through proteolytic cleavage or by reduction of disulfide bond, but not before the ADC reaches its target[44]. Furthermore, the antibodies stay in the surface of the cells and can be accessible to effector cells of the immune system, activating the antibody-dependent cellular cytotoxicity (ADCC), a mechanism that lyse the cells. This can further reinforce the action of the drug. This mechanism includes a wider selection of targets, including non-internalizing receptors, which may be beneficial in increasing efficacy, but may also lead to lower cell-selectivity and off-target toxicity.

3.2 Internalization and trafficking Increasing methods

As the efficacy of most ADCs depends on lysosomal degradation for releasing and activating the drug, except for ADCs that release their target outside the cell, they require efficient internalization and trafficking to lysosomal compartments. Some ADC targets though, like human epidermal growth factor receptor 2 (HER2) that is overexpressed in breast and gastric tumor cells, except for internalizing into the cell, it can also be recycled back to the plasma membrane before it reaches the lysosomes, thus ADC remains inactive. Bispecific antibody approach has given the possibility to increase successful lysosomal trafficking either by targeting fast internalization receptors together with the target antigen, or by

crosslinking and clustering of the receptors[18].

3.2.1 Fast-internalizing receptors

In the first approach, bispecific ADC targets a cell receptor that promotes trafficking to the lysosomes, so that the drug can reach lysosomes and be released. At the same time, it targets a different cell surface antigen that is overexpressed and provides selectivity to the specific target. This dual targeting results both in efficacy and selectivity of the design ADC[45]. Many trials combine the HER2 receptor, with rapid internalizing receptors to "drag" it to the lysosomes. Prolactin Receptor (PRLP), is a representative example, as it constitutively traffics to lysosomes and is rapidly degraded. It is implicated in breast cancer and it is expressed in breast tumor cell. A bispecific ADC binding both HER2 and RPLP, showed an enhanced HER2 degradation and drug effectiveness in breast cancer cells that coexpress HER2 and PRLR, compared to HER2 monospecific ADC. This bsADC contained a HER2 arm from trastuzumab, that is conjugated to the tubulin inhibitor DM1, and a PRLR arm from a monospecific antibody. Confocal microscopy showed its internalization route inside the cancer cells and confirmed the efficient lysosomal trafficking, while cell viability assay was performed for both bispecific and monospecific ADCs for each target[46].

3.2.2 Cross-linking and clustering

In the second approach, a biparatopic antibody is constructed, which recognizes two non-overlaping epitopes of the target antigen and can induce clustering and cross-linking of receptors. A tetravalent birapatopic antibody conjugated with a tubulysin-based microtubule inhibitor was designed to target HER2 receptor. Due to its tetravalent binding capacity it was able to cross-link HER2, forming large groups of receptors. This clustering promotes internalization, lysosomal trafficking and degradation, which activates the drug and leads to enhance anti-tumor activity, validated by measurements of growth inhibition percentages[47].

3.3 Linker design

The linker that connects the antibody with the drug is one of the major parts to be considered in BsADC design. As it was previously discussed, when the BsADC is administrated, it travels through the body to reach its target and following endocytosis it can pass by different compartments of the cell. In order to stay conjugated in all of these different environments so that the drug is not released and activated prior to reaching its target cell, the linker has to be stable outside the cells so that the drug is optimally released inside or on the surface of the cell. There are two types of linkers that are used based on the mechanism of the drug release: non-cleavable and cleavable linkers[22],[20].

3.3.1 Non-cleavable linkers

Non-cleavable linkers are resistant to proteolytic degradation and provide greater stability than the cleavable linkers. In principle, a drug ligated with an antibody by this type of linker can only be released after the complete degradation of the antibody in the lysosome or the cytosol by proteases, like aspartic, cysteine, or serine proteinase family. In this case, the drug remains with the linker and the amino acid by which it was conjugated with the antibody. Having this in mind, the structure has to be designed such that the drug is still active with the linker conjugated[48]. A successful example of a

monospecific ADC conjugated via a stable linker is Trastuzumab Emtansine (T-DM1). This antibody recognizes human epidermal growth factor receptor (HER2) on breast tumor cells and transfers the DM1 cytotoxic drug which is a derivative of mertansine and acts as a tubulin inhibitor. DM1 is linked to trastuzumab via a non-reducible thioether linkage from which is released and activated only intracellularly after antibody lysosomal degradation[49].

3.3.2 Cleavable linkers

Cleavable linkers are the major type of ADC linkers, due to their range of applicability as they present multiple ways of releasing the drug. To accomplish this, they exploit differences in extracellular and intracellular environmental conditions, such as differences in pH and lysosomal enzymes. According to this, they are categorized as chemically-cleavable or enzyme-cleavable linkers[22].

Chemical-cleavable linkers

Chemical-cleavable linkers can be distinguished in acid cleavable, reducible disulfides and those cleaved by exogenous stimuli.

Acid cleavable linkers, like hydrazine linkers (figure 5), are sensitive to the acidity of endosomes (pH 5.5.-6.2) and/or lysosomes (pH 4.5-5.0) but stable in the alkaline environment of systemic circulation (pH 7.4). Upon internalization in endosomes or lysosomes, the hydrazone group of the linker gets hydrolyzed, leading to linker cleavage and drug release. This type of linker has been used in two ADCs that are clinically approved and exist in the market, the gemtuzumab ozogamicin (Mylotarg), which targets CD33 receptor on the acute myeloid leukemia cells and carries the antitumor antibiotic calicheamicin[50], and the inotuzumab ozogamicin (Besponsa), which targets the CD22+ antigen on B cells in patients with B-Acute lymphoblastic leukemia and transfers calicheamicin. In vivo and in vitro stability of these linkers was tested in mice and showed a very small rate of hydrazine hydrolysis in circulation.[51]. Besides the clinical success of these ADCs, the requirement of strictly discrimination between pH values makes their design difficult and in some clinical trials leads to non-specific release of the drug[52],[22].

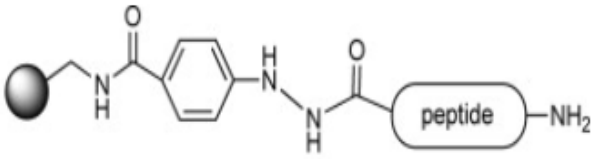

Figure 5: Hydrazine linker between a peptide and a bead[53].

Reducible disulfide linkers on the other hand, are sensitive to the nucleophilic attack from cytosolic thiols and especially from glutathione (figure 6), while they remain stable in the oxidizing conditions in the circulation and tissues. Glutathione is a small molecular tripeptide that contains thiol groups and is present in elevated levels in the cytosol during tumor growth and hypoxic cell conditions to protect against oxidative stress. Thus, its concentration can be higher in cancer cells than in normal cells. Clinical success has been found in Mylotarg and Besponsa ADCs alongside hydrazines[52],[22].

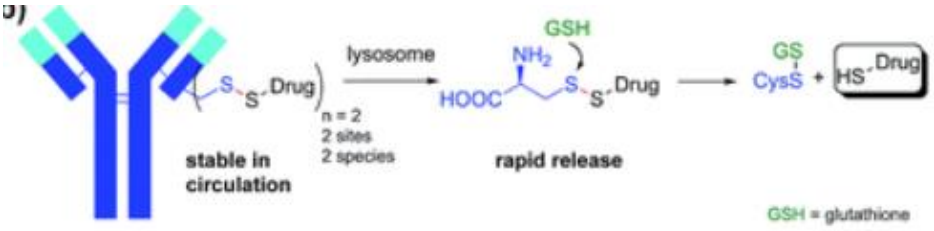

Figure 6: Drug release after cleavage of the disulfide linker from glutathione[54].

The last category of chemical-cleavable linkers includes linkers that are cleaved outside of the cell via administrated external molecules that trigger drug release. To eliminate early release of the drug, the external stimulators are administrated to the body hours or days later than the ADC, so that the ADC has enough time to bind to the target[52]. A trans-cyclooctene linker in a non-internalizing ADC has been tested to react with external administrated tetrazine activator, releasing a monomethyl autistatin E (MMAE) payload in vivo (figure 7) and eventually displaying great antitumor activity in mice. Biodistribution and imaging studies in mouse xenograft models confirmed the high tumor uptake of the antibody and the released of drug by the tumor activator, with very low levels of non-target tissues[55].

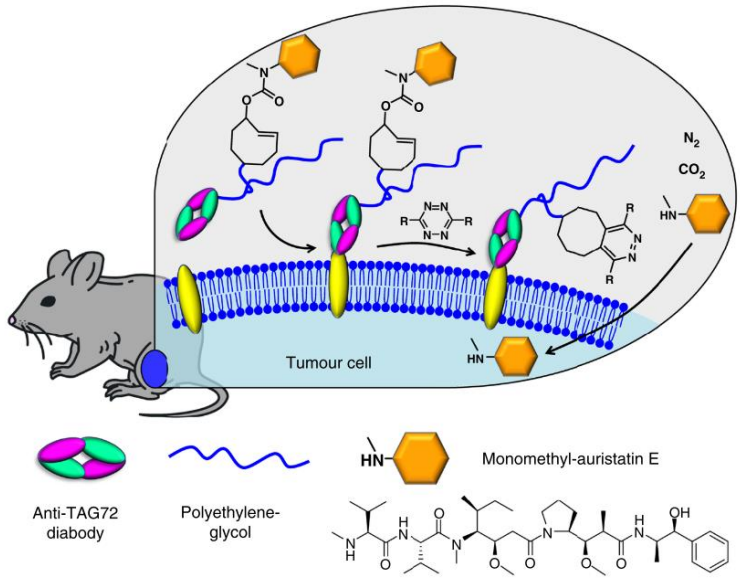

Figure 7: Triggered drug release using “click-to-release” chemistry in vivo: on-tumour liberation of a cell permeable drug (monomethyl auristatin E, MMAE) from a trans-cyclooctene-linked ADC following systemic administration of a tetrazine activator[55].

Enzyme-cleavable linkers

The category of enzyme-cleavable linkers contains peptide-based, β-glucuronidase and phosphatase-cleavable linkers.

Peptide-based linkers are used in the majority of ADCs in clinical trials and are stable in systematic circulation but sensitive to lysosomal protease activity. Compared to healthy cells, tumor cells can exhibit higher expression of lysosomal proteases like cathepsin B, which can be secreted into the extracellular matrix and facilitate its destruction, thus be correlated with invasive and metastatic

phenotypes[56]. This protease overexpression increases the selectivity of the drug for cancer cells. Valine-cirtulline dideptide linker is the most commonly used dipeptide-linker in ADC design and has been applied in the FDA-approved ADC Adcentris (Brentuximab Vedotin), the first approved drug for Hodgkin lymphoma. It recognizes the CD30 antigen expressed on Hodgkin lymphoma and anaplastic cell lymphoma and it is attached to mono-methylauristatin E (MMAE), a potent inhibitor of microtubule polymerization (figure 8). Anti-idiotype and antidrug mAbs captured and quantified in vitro the anti-CD30 antibody and MMAE drug respectively, following its release by cathepsin B, while in vivo studies in immunodeficient mice and cynomolgus monkeys revealed linker's high stability in blood circulation [57,58].

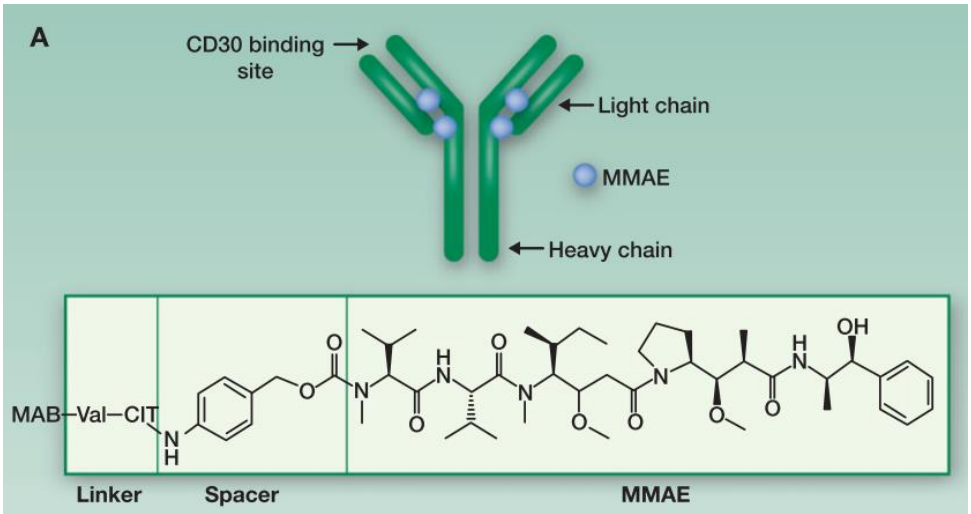

Figure 8: Structure of the Brentuximab Vedotin ADC[58].

β-Glucuronidase-cleavable linkers contain β-glucuronic acid substrates which are hydrolyzed by β-glucuronidases. This lysosomal enzyme is produced by tumor cells and has extracellular activity, which has promoted the application of β-glucuronidase-cleavable linkers in ADCs. Similarly, β-galactosidase-cleavable linkers have been designed, which are hydrolyzed by a homologous protein to β-glucuronidase enzyme, β-galactosidase, which is overexpressed in certain tumor types. Both linkers have been tested in ADCs in isolated rat plasma and were found more stable compared to the dipeptide linkers. The payload's release in antigen positive cells, was found efficient after in vitro testing by analysis of cell extraction[52].

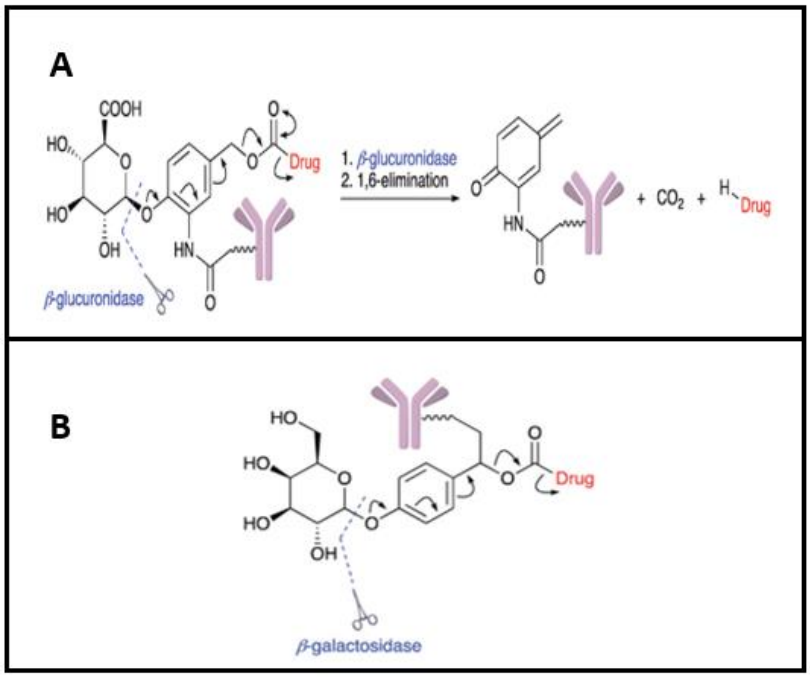

Figure 9: The structure and release mechanism of A) β-glucuronic acid-containing ADC and B) β-galactoside-containing ADC.

Last, phosphatase-cleavable linkers can be hydrolyzed by phosphatases in lysosomes. Phosphate and pyrophosphate groups have been incorporated into linkers into ADCs so that the drug is released after their hydrolysis (figure 10). Drug release was tested in lysosomal extract in vitro, demonstrated that the pyrophosphate bearing linker released the payload more rapidly than the monophosphate bearing linker, making the ADC more potent. Moreover, ADCs with pyrophosphate bearing linker was tested in mouse and human plasma and full stability was observed over seven days[52].

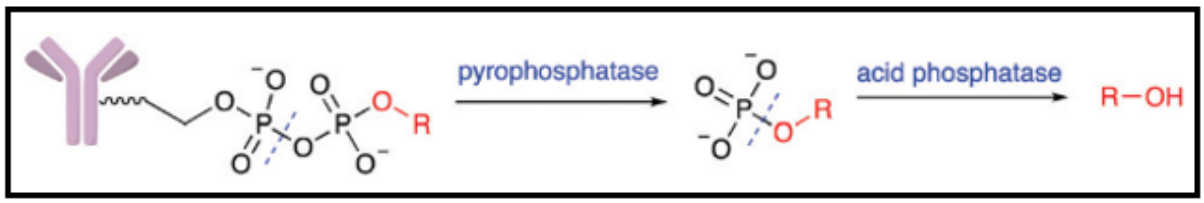

Figure 10: The structure of cleavage mechanism of pyrophosphate-containing ADCs bearing payloads (R)[52]

3.4 Antibody-drug pre-conjugation method

The main strategy to produce an ADC is by conjugating the antibody and the drug by a linker and then inject it into the body. The synthesis of an ADC requires the selection of the most suitable linker, as was described above, and the appropriate conjugation method. One of the most commonly used method is the conjugation via thiols, that can be found in one of the interchain disulfide bonds of antibodies. After the reduction of the disulfide bonds, the thiols are accessible for the linker with the drug to form a new bond and conjugate to the antibody[60]. Because antibodies can have multiple disulfide bonds, more than one drug moiety can be attached to them, as long as they don't disturb antibody's structure. An ADC

consisting of monomethyl auristatin E conjugated to the anti-CD30 monoclonal antibody have been designed to contain several drug moieties per mAb, coupled to the cysteines that comprise the interchain disulfide bonds. The maximum number of drugs per antibody that has been obtained is eight (E8), whereas the number that shows the best in vivo performance four (E4). Specifically, the antitumor activity of both ADCs was equal, as obtained from in vivo studies, but the maximum-tolerated dose in mice was double in E4 than in E8 and its clearance from the plasma was 3-folds faster in E8 than in E4[61]. Alternative methods for linking the payload and the antibody are conjugation via Amines, alcohols or aldehydes from the side chain of the antibody's amino acids[60].

3.5 Ways of administration

BsADCs are usually intravenously injected in the blood circulation, by which they can travel through the body and reach their target. There are two applied methods by which the drug reaches the target in association with the bispecific antibody. Either by conjugation with the antibody before injection as discussed in the previous paragraph or by pretargeted delivery of the bsAb and afterword delivery of the drug[59].

Pretargeted delivery

The pretargeting technique gives the advantage of rapid elimination of unbound drug in the organism, minimizing its toxicity. Certain ADCs, like radiolabeled antibodies, where the drug cannot be inactivated for their transport to the target, show a slow blood clearance in the body and as a result continuous radiation exposure of healthy tissues. With the pretargeting method, a bsAb with affinity for a target cell and a hapten, a small molecule that acts like an antigen but is not immunogenic, is first administrated into the body and binds to the target while the unbound molecules clear from the circulation. Then a radiolabeled hapten peptide is granted and is captured by the antibody (figure 11), while the remainder is cleared very quickly from the blood due to its small size[62].
The first clinical trial with this pretargeting method was applied for the treatment of advanced colorectal cancer, by the use of a trivalent bispecific antibody with affinity for two cancer epitopes, CEA and CEM5, and for the ^{111}indium radiolabeled hapten-peptide, IMP288. The bsAb and the peptide was tested in patients with metastatic colorectal cancer in terms of time of administration and molecular concentration. The optimal tumor targeting was observed with a one-day interval between the bsAb and the peptide, while the latest was visualized to reach the bsAb within one hour after its administration and with high tumour-to-tissue ratio[63].

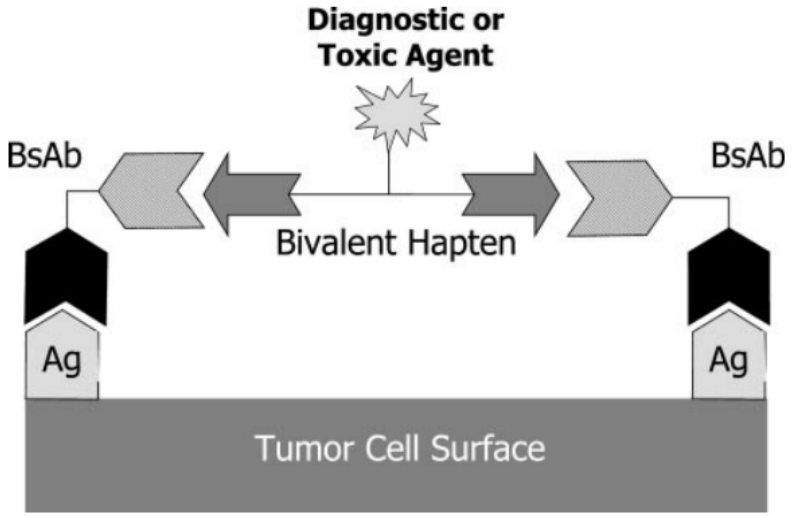

Figure 11: A bivalent hapten that carries a diagnostic or toxic agent and can bind and cross-link 2 bsAbs in vivo on the targeted tumor surface.

4. Discussion

Bispecific antibodies have been a very promising tool to expand the therapeutic application of antibodies and create new generation ADCs. The wide range of possibilities in terms of design and mechanism of action provides flexibility in the generation of novel therapeutic and diagnostic agents in an attempt to advance modern medicine. In combination with other technologies, new bsADCs can be generated directed against a variety of diseases for which therapeutic approaches are still inefficient, like many types of cancer, or for the development of next generation diagnostic methods. Although this seems to be a promising and evolving technology, a lot of work is still needed in engineering such molecules to obtain the best and well suited characteristics for each application and perform optimally. For example, testing of pharmacological properties, like pharmacokinetics and pharmacodynamics, is essential before their application.

5. Bibliography

1. Chaplin, D. D. Overview of the Immune Response. **845**, 35294–2170.
2. Beenhouwer, D. O. Molecular basis of diseases of immunity. in *Molecular Pathology: The Molecular Basis of Human Disease* 329–345 (Elsevier Inc., 2018). doi:10.1016/B978-0-12-802761-5.00017-1.
3. Ribatti, D. Edelman's view on the discovery of antibodies. *Immunology Letters* vol. 164 72–75 (2015).
4. Padlan, E. A. Structural basis for the specificity of antibody-antigen reactions and

structural mechanisms for the diversification of antigen-binding specificities. *Q. Rev. Biophys.* **10**, 35–65 (1977).

5. Chiu, M. L., Goulet, D. R., Teplyakov, A. & Gilliland, G. L. Antibody Structure and Function: The Basis for Engineering Therapeutics. *Antibodies* **8**, 55 (2019).

6. Smith, S. L. Ten Years of Orthoclone OKT3 (Muromonab-CD3): A Review. *J. Transpl. Coord.* **6**, 109–121 (1996).

7. Morrison, S. L., Johnsont, M. J., Herzenbergt, L. A. & 01i, V. T. *Chimeric human antibody molecules: Mouse antigen-binding domains with human constant region domains (transfection/protoplast fusion/calcium phosphate transfection/intronic controlling elements/transfectoma). Proc. Nati. Acad. Sci. USA* vol. 81 (1984).

8. Green, L. L. *et al.* Antigen–specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs. *Nat. Genet.* **7**, 13–21 (1994).

9. Khoshnejad, M. *et al.* Molecular engineering of antibodies for site-specific covalent conjugation using CRISPR/Cas9. *Sci. Rep.* **8**, 1–9 (2018).

10. Van Der Schoot, J. M. S. *et al.* Functional diversification of hybridoma-produced antibodies by CRISPR/HDR genomic engineering. *Sci. Adv.* **5**, 1–11 (2019).

11. Wu, Z. & Guo, Z. Sortase-mediated transpeptidation for site-specific modification of peptides, glycopeptides, and proteins. *J. Carbohydr. Chem.* **31**, 48–66 (2012).

12. Search of: Antibodies, Monoclonal - List Results - ClinicalTrials.gov. https://clinicaltrials.gov/ct2/results?intr=Antibodies%2C+Monoclonal&age_v=&gndr=&type=&rslt=&Search=Apply.

13. Suzuki, M., Kato, C. & Kato, A. Therapeutic antibodies: Their mechanisms of action and the pathological findings they induce in toxicity studies. *J. Toxicol. Pathol.* **28**, 133–139 (2015).

14. Aalberse, R. C. & Schuurman, J. IgG4 breaking the rules. *Immunology* vol. 105 9–19 (2002).

15. Search of: Antibodies, Bispecific Monoclonal - List Results - ClinicalTrials.gov. https://clinicaltrials.gov/ct2/results?intr=Antibodies%2C+Bispecific+Monoclonal&age_v=&gndr=&type=&rslt=&Search=Apply.

16. Lu, R. M. *et al.* Development of therapeutic antibodies for the treatment of diseases. *J. Biomed. Sci.* **27**, 1–30 (2020).

17. Nie, S. *et al.* Biology drives the discovery of bispecific antibodies as innovative therapeutics. *Antib. Ther.* **3**, 18–62 (2020).

18. Maruani, A. Bispeci fi cs and antibody – drug conjugates : A positive synergy. **xxx**, 1–7 (2018).

19. Strebhardt, K. & Ullrich, A. Paul Ehrlich's magic bullet concept: 100 Years of progress. *Nature Reviews Cancer* vol. 8 473–480 (2008).

20. Wilson, R. Antibody Drug Conjugates: New Weapons Against Cancer. *Oncol. Times* **42**, 12–13 (2020).

21. Zafir-Lavie, I., Michaeli, Y. & Reiter, Y. Novel antibodies as anticancer agents. *Oncogene* vol. 26 3714–3733 (2007).

22. Khongorzul, P., Ling, C. J., Khan, F. U., Ihsan, A. U. & Zhang, J. *Antibody-drug conjugates: A comprehensive review*. *Molecular Cancer Research* vol. 18 (2020).

23. Wang, Q. *et al.* Design and Production of Bispecific Antibodies. *Antibodies* **8**, 43 (2019).

24. Kontermann, R. E. & Brinkmann, U. Bispecific antibodies. *Drug Discov. Today* **20**, 838–847 (2015).

25. Brinkmann, U. & Kontermann, R. E. The making of bispecific antibodies. *MAbs* **9**, 182–212 (2017).

26. Holliger, P., Prospero, T. & Winter, G. 'Diabodies': Small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 6444–6448 (1993).

27. Johnson, S. *et al.* Effector cell recruitment with novel Fv-based dual-affinity re-targeting protein leads to potent tumor cytolysis and in vivo B-cell depletion. *J. Mol. Biol.* **399**, 436–449 (2010).

28. Reusch, U. *et al.* A novel tetravalent bispecific TandAb (CD30/CD16A) efficiently recruits NK cells for the lysis of CD30+ tumor cells. *MAbs* **6**, 727–738 (2014).

29. Moldenhauer, G. Bispecific Antibodies from Hybrid Hybridoma. in *Bispecific Antibodies* 29–46 (Springer Berlin Heidelberg, 2011). doi:10.1007/978-3-642-20910-9_2.

30. Ridgway, J. B. B., Presta, L. G. & Carter, P. 'Knobs-into-holes' engineering of antibody C(H)3 domains for heavy chain heterodimerization. *Protein Eng.* **9**, 617–621 (1996).

31. Gunasekaran, K. *et al.* Enhancing Antibody Fc Heterodimer Formation through Electrostatic Steering Effects. *J. Biol. Chem.* **285**, 19637–19646 (2010).

32. M, M. *et al.* Therapeutic Assessment of SEED: A New Engineered Antibody Platform Designed to Generate Mono- And Bispecific Antibodies. *Protein Eng. Des. Sel.* **24**, (2011).

33. Moore, G. L. *et al.* A robust heterodimeric Fc platform engineered for efficient

development of bispecific antibodies of multiple formats. *Methods* **154**, 38–50 (2019).

34. Shiraiwa, H. *et al.* Engineering a bispecific antibody with a common light chain: Identification and optimization of an anti-CD3 epsilon and anti-GPC3 bispecific antibody, ERY974. *Methods* **154**, 10–20 (2019).

35. Klein, C. *et al.* Engineering therapeutic bispecific antibodies using CrossMab technology. *Methods* **154**, 21–31 (2019).

36. Spiess, C., Zhai, Q. & Carter, P. J. Alternative molecular formats and therapeutic applications for bispecific antibodies. *Molecular Immunology* vol. 67 95–106 (2015).

37. Klein, C., Schaefer, W. & Regula, J. T. The use of CrossMAb technology for the generation of bi- and multispecific antibodies. *mAbs* vol. 8 1010–1020 (2016).

38. Grant, B. D. & Donaldson, J. G. Pathways and mechanisms of endocytic recycling. *Nature Reviews Molecular Cell Biology* vol. 10 597–608 (2009).

39. Kovtun, Y. V. & Goldmacher, V. S. Cell killing by antibody-drug conjugates. *Cancer Lett.* **255**, 232–240 (2007).

40. Kaksonen, M. & Roux, A. Mechanisms of clathrin-mediated endocytosis. *Nat. Rev. Mol. Cell Biol.* **19**, 313–326 (2018).

41. Kiss, A. L. & Botos, E. Endocytosis via caveolae: Alternative pathway with distinct cellular compartments to avoid lysosomal degradation? *J. Cell. Mol. Med.* **13**, 1228–1237 (2009).

42. Law, C. L. *et al.* Efficient elimination of B-lineage lymphomas by anti-CD20-auristatin conjugates. *Clin. Cancer Res.* **10**, 7842–7851 (2004).

43. Smith, L. M., Nesterova, A., Alley, S. C., Torgov, M. Y. & Carter, P. J. Potent cytotoxicity of an auristatin-containing antibody-drug conjugate targeting melanoma cells expressing melanotransferrin/p97. *Mol. Cancer Ther.* **5**, 1474–1482 (2006).

44. Casi, G. & Neri, D. Noninternalizing targeted cytotoxics for cancer therapy. *Mol. Pharm.* **12**, 1880–1884 (2015).

45. Lee, J. M. *et al.* Novel strategy for a bispecific antibody: Induction of dual target internalization and degradation. *Oncogene* **35**, 4437–4446 (2016).

46. Andreev, J. *et al.* Bispecific antibodies and antibody-drug conjugates (ADCs) bridging HER2 and prolactin receptor improve efficacy of HER2 ADCs. *Mol. Cancer Ther.* **16**, 681–693 (2017).

47. Li, J. Y. *et al.* A Biparatopic HER2-Targeting Antibody-Drug Conjugate Induces Tumor Regression in Primary Models Refractory to or Ineligible for HER2-Targeted Therapy.

Cancer Cell **29**, 117–129 (2016).

48. Tsuchikama, K. & An, Z. Antibody-drug conjugates: recent advances in conjugation and linker chemistries. *Protein and Cell* vol. 9 33–46 (2018).

49. LoRusso, P. M., Weiss, D., Guardino, E., Girish, S. & Sliwkowski, M. X. Trastuzumab emtansine: A unique antibody-drug conjugate in development for human epidermal growth factor receptor 2-positive cancer. *Clinical Cancer Research* vol. 17 6437–6447 (2011).

50. Hamann, P. R. *et al.* Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody - Calicheamicin conjugate for treatment of acute myeloid leukemia. *Bioconjug. Chem.* **13**, 47–58 (2002).

51. DiJoseph, J. F. *et al.* Antibody-targeted chemotherapy with CMC-544: A CD22-targeted immunoconjugate of calicheamicin for the treatment of B-lymphoid malignancies. *Blood* **103**, 1807–1814 (2004).

52. Bargh, J. D., Isidro-Llobet, A., Parker, J. S. & Spring, D. R. Cleavable linkers in antibody-drug conjugates. *Chem. Soc. Rev.* **48**, 4361–4374 (2019).

53. Görmer, K., Waldmann, H. & Brunsveld, L. Lipidation of peptides and proteins. in *Comprehensive Natural Products II: Chemistry and Biology* vol. 5 531–585 (Elsevier Ltd, 2010).

54. Pillow, T. H. *et al.* Decoupling stability and release in disulfide bonds with antibody-small molecule conjugates. *Chem. Sci.* **8**, 366–370 (2016).

55. Rossin, R. *et al.* Chemically triggered drug release from an antibody-drug conjugate leads to potent antitumour activity in mice. *Nat. Commun.* **9**, (2018).

56. Gondi, C. S. & Rao, J. S. Cathepsin B as a cancer target. *Expert Opinion on Therapeutic Targets* vol. 17 281–291 (2013).

57. Sanderson, R. J. *et al.* In vivo drug-linker stability of an anti-CD30 dipeptide-linked auristatin immunoconjugate. *Clin. Cancer Res.* **11**, 843–852 (2005).

58. Katz, J., Janik, J. E. & Younes, A. Brentuximab vedotin (SGN-35). *Clinical Cancer Research* vol. 17 6428–6436 (2011).

59. Suurs, F. V., Lub-de Hooge, M. N., de Vries, E. G. E. & de Groot, D. J. A. A review of bispecific antibodies and antibody constructs in oncology and clinical challenges. *Pharmacol. Ther.* **201**, 103–119 (2019).

60. Yao, H., Jiang, F., Lu, A. & Zhang, G. Methods to design and synthesize antibody-drug conjugates (ADCs). *International Journal of Molecular Sciences* vol. 17 (2016).

61. Hamblett, K. J. *et al.* Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. *Clin. Cancer Res.* **10**, 7063–7070 (2004).

62. Chang, C.-H. *et al.* Molecular advances in pretargeting radioimunotherapy with bispecific antibodies. *Mol. Cancer Ther.* **1**, 553–63 (2002).

63. Schoffelen, R. *et al.* Development of an imaging-guided CEA-pretargeted radionuclide treatment of advanced colorectal cancer: First clinical results. *Br. J. Cancer* **109**, 934–942 (2013).

64. Altai, M., Membreno, R., Cook, B., Tolmachev, V. & Zeglis, B. M. Pretargeted imaging and therapy. *J. Nucl. Med.* **58**, 1553–1559 (2017).