ANTIBODY-DRUG CONJUGATES: A SYSTEMATIC REVIEW

Bostyn Lisa

A Master dissertation for the study programme Master in Drug Development

Academic year: 2020 - 2021
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PREAMBLE
This master's thesis was executed in a period where corona measures have influenced research and education activities in various ways. These unusual circumstances may have had an impact on this thesis to a greater or lesser extent, despite all the efforts of the student, daily supervisor(s) and promoters. This generic preamble aims to frame this and was approved by the faculty.
SUMMARY

Antibody-drug conjugates (ADCs) are a relatively new class of biological therapeutics marrying a highly potent drug alongside the exceptional specificity of an antibody with an eye towards improving the therapeutic index of the former. This 100-year-old concept was met with limited success at first. However, progress with regard to different components of the conjugate over the past few decades has catalysed the development, clinical evaluation and breakthrough of ADCs with two conjugates receiving approval in 2019 and three more in 2020. Currently, there are 9 ADCs on the market in the battle against various cancers, both solid and haematological.

In this literature review it is aimed to collect all acquired knowledge of the past years and, on the strength of this systematic review, an overview is offered on ADC design, mechanism of action, toxicity profiles and more. More in detail, several IgG antibody backbones were discussed and their potential replacement by smaller and/or bispecific targeting ligands. This could significantly enhance tumour penetration, especially in solid tumours, with the prospect of an increased, more homogeneous drug distribution and ultimately complete tumour killing.

Diverse strategies for a controlled, homogeneous drug linker conjugation to the antibody backbone were summarised. This is important as naked antibodies can act as competitive inhibitors whereas overloaded conjugates may cause aggregation and an overall pharmacological disadvantage. Furthermore, the most commonly used linkers and payloads (the cytotoxic drug) were listed. In practise, a cleavable and lipophilic payload is often chosen to promote the so-called “bystander effect”. This involves diffusion of the hydrophobic payload from the target cell (after internalisation) into adjacent cells (including, antigen-negative cells), benefitting tumour heterogeneity. Cleavable linkers are designed to decompose in reaction to the inherent properties of the tumour, whereas non-cleavable linkers depend on lysosomal proteolytic degradation. This often leaves a charged amino acid on the free payload affecting its membrane permeability, however, in some cases they are preferred due to a superior plasma stability. In addition, their mechanism of action, clinical activity, toxicity profiles and resistance were covered. Finally, an overview of the ADCs currently on the market and their characteristics is given. A recurring conclusion in each of these chapters is certainly the "one size does not fit all" principle, thus future ADC design should focus more on multi-parameter optimisation, taking into account the total construct of the ADC.

In conclusion, this literature study provides a complete insight into the antibody-drug conjugate concept for anyone with interest of exploring this promising drug class.
SAMENVATTING

Antibody-drug conjugates (ADCs) zijn een nieuwe klasse aan biotherapeutica waarbij een uiterst krachtig geneesmiddel gekoppeld wordt met de uitzonderlijke specificiteit van een antilichaam, met het oog op een nauwere therapeutische index in vergelijking met klassieke chemotherapeutica. Deze werken ook vaak in op gezonde cellen. Dit 100 jaar oude concept, hoewel vernieuwend en veelbelovend, had aanvankelijk slechts een beperkt succes. Sindsdien is er vooruitgang geboekt, dit omdat er de afgelopen decennia waardevol onderzoek is verricht naar elk onderdeel van het conjugaat, terwijl tumorbiologie steeds verder wordt ontrafeld. ADCs lijken eindelijk door te breken aangezien twee conjugaten goedkeuring hebben gekregen in 2019 en nog eens drie in 2020. Momenteel zijn er 9 ADCs op de markt in de strijd tegen verschillende, zowel solide als hematologische, kankers. In dit literatuuronderzoek werd ernaar gestreefd om alle verworven kennis van de afgelopen jaren te verzamelen en op basis van deze systematische review een overzicht te bieden van ADC ontwerp, werkingsmechanisme, toxiciteitsprofielen en meer.

Meer concreet werden de verschillende subtypes aan IgG antilichamen besproken en hun mogelijke vervanging door kleinere en/of bespecifieke formaten. Hierdoor zou tumor penetratie verbeterd kunnen worden, vooral in solide tumoren, met het oog op een hogere, meer homegene geneesmiddel distributie en uiteindelijk een algehele afdoding van het tumorgezwel. Verschillende methoden voor een meer gecontroleerde en homogene drug linker conjugatie aan het antilichaam werden ook samengevat. Dit is belangrijk want ongeconjugeerde antilichamen kunnen fungeren als competitieve inhibitoren, terwijl overladen conjugaten aggregatie kunnen veroorzaken, resulterend in een algemeen farmacologisch nadeel. Verder werden de meest gebruikte linkers en payloads (het cytotoxisch geneesmiddel) opgelijst. Vaak wordt er echter gekozen voor een 'cleavable' linker en een lipofiele payload, dit om het zogenaamde 'bystander effect' te bevorderen. Hierbij kan de payload na internalisatie uit de tumorcel diffunderen naar aangrenzende cellen (waaronder antigen-negatieve cellen), ten voordele van heterogene tumoren. Cleavable linkers zijn ontworpen voor afbraak door de inherente eigenschappen van de tumor, waarnaast non-cleavable linkers enkel proteolytisch worden afgebroken in de lysosomen. Hierdoor blijft vaak een geladen aminozuur over op de vrije payload wat zijn permeabiliteit zal beïnvloeden, toch worden deze soms verkozen voor hun betere plasma stabiliteit. Daarnaast werd er dieper ingegaan op de klinische resultaten van deze conjugaten, hun toxiciteitsprofiel en resistentiemechanismen. Er wordt afgesloten met een overzicht van de ADCs momenteel op de markt en hun kenmerken. Een terugkerend besluit bij elk van deze hoofdstukken is zeker het "one size does not fit all" principe, toekomstig ADC design moet meer inzetten op multi-parameter optimalisering, rekening houdend met het totale construct van de ADC. Deze literatuurstudie biedt een compleet overzicht van het antibody-drug conjugate concept voor iedereen die zich hierin wil verdiepen.
ACKNOWLEDGEMENTS

During an intensive four-month project, I conducted a review on the intriguing concept of antibody-drug conjugates and wrote my thesis on it. It was a period in which I learned to set up a valid and reliable research, as well as how to better evaluate and improve my work. Needless to say, I did not achieve this by myself. Therefore, I would like to express my gratitude to the persons who took their time to support and advise me.

First and most importantly, I would like to thank my promoter Prof. Dr. Apr. Van Calenbergh, this thesis would not have come to a successful end without his guidance and invaluable input. For me, it was a truly captivating literature review that I enjoyed working on.

Second, I would also like to thank Ghent University and their attentive staff for the personal study place I got in Urbis despite the corona epidemic. A special thank you to my faculty for allowing the theses to continue as normal as possible even in a time like this.

Furthermore, I would like to acknowledge my parents for supporting my studies and always helping me when needed. Lastly, I am most appreciative for my friends and boyfriend who provided the fun moments alongside my thesis.
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<th>Description</th>
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<tbody>
<tr>
<td>AAZ</td>
<td>acetazolamide</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette transporters</td>
</tr>
<tr>
<td>ABD</td>
<td>albumin-binding domain</td>
</tr>
<tr>
<td>ADA</td>
<td>antidrug antibodies</td>
</tr>
<tr>
<td>ADC</td>
<td>antibody-drug conjugates</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>ADCP</td>
<td>antibody-dependent cellular phagocytosis</td>
</tr>
<tr>
<td>bsADC</td>
<td>bispecific antibody-drug conjugates</td>
</tr>
<tr>
<td>CA-IX</td>
<td>carbonic anhydrase</td>
</tr>
<tr>
<td>cBu</td>
<td>cyclobutane-1,1-dicarboxamide</td>
</tr>
<tr>
<td>CDC</td>
<td>complement-dependent cytotoxicity</td>
</tr>
<tr>
<td>CPP</td>
<td>cell-penetrating peptides</td>
</tr>
<tr>
<td>CuAAC</td>
<td>copper(I)-catalysed azide-alkyne cycloaddition</td>
</tr>
<tr>
<td>DAR</td>
<td>drug-to-antibody ratio</td>
</tr>
<tr>
<td>DM</td>
<td>drug maytansinoids</td>
</tr>
<tr>
<td>Fab</td>
<td>antigen binding fragment</td>
</tr>
<tr>
<td>Fc</td>
<td>crystallizable fragment</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc gamma receptor</td>
</tr>
<tr>
<td>FcRn</td>
<td>neonatal Fc receptor</td>
</tr>
<tr>
<td>FGE</td>
<td>formylglycine-generating enzyme</td>
</tr>
<tr>
<td>GalNAz</td>
<td>N-azidoacetyl-galactosamine</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigens</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>LRPI</td>
<td>lipoprotein receptor-related protein 1</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>mc</td>
<td>maleimidocaproyl</td>
</tr>
<tr>
<td>mcc</td>
<td>maleimidomethyl cyclohexane-1-carboxylate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MMAE</td>
<td>monomethyl auristatin E</td>
</tr>
<tr>
<td>MMAF</td>
<td>monomethyl auristatin F</td>
</tr>
<tr>
<td>MOA</td>
<td>mechanism of action</td>
</tr>
<tr>
<td>mTGase</td>
<td>microbial transglutaminase</td>
</tr>
<tr>
<td>N297</td>
<td>asparagine 297</td>
</tr>
<tr>
<td>ORR</td>
<td>objective response rate</td>
</tr>
<tr>
<td>PAB</td>
<td>para-aminobenzyl</td>
</tr>
<tr>
<td>PABC</td>
<td>para-aminobenzylcarbamate</td>
</tr>
<tr>
<td>pAcPhe</td>
<td>para-acetylphenylalanine</td>
</tr>
<tr>
<td>pAMF</td>
<td>para-azidomethyl-l-phenylalanine</td>
</tr>
<tr>
<td>PBD</td>
<td>pyrrolobenzodiazepine</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PK</td>
<td>pharmacokinetic</td>
</tr>
<tr>
<td>PK/PD</td>
<td>pharmacokinetic/pharmacodynamic</td>
</tr>
<tr>
<td>PLK1</td>
<td>polo-like kinase 1</td>
</tr>
<tr>
<td>R/R</td>
<td>relapsed and/or refractory</td>
</tr>
<tr>
<td>S.A.R.</td>
<td>structure–activity relationship</td>
</tr>
<tr>
<td>scFv</td>
<td>single chain variable fragments</td>
</tr>
<tr>
<td>SMDC</td>
<td>small-molecule-drug conjugate</td>
</tr>
<tr>
<td>SPAAC</td>
<td>strain-promoted azide–alkyne cycloaddition</td>
</tr>
<tr>
<td>SPARC</td>
<td>secreted protein acidic and rich in cysteine</td>
</tr>
<tr>
<td>T-DM1</td>
<td>trastuzumab emtansine</td>
</tr>
<tr>
<td>T-DXd</td>
<td>trastuzumab deruxtecan</td>
</tr>
<tr>
<td>tCPP</td>
<td>tetrameric cell-penetrating peptides</td>
</tr>
<tr>
<td>TDM</td>
<td>therapeutic drug monitoring</td>
</tr>
<tr>
<td>TME</td>
<td>tumour microenvironment</td>
</tr>
<tr>
<td>TOP01</td>
<td>topoisomerase 1</td>
</tr>
<tr>
<td>UAA</td>
<td>unnatural amino acids</td>
</tr>
<tr>
<td>VEGFA</td>
<td>vascular endothelial growth factor A</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

Antibody-drug conjugates (ADCs) are a promising new drug class in oncology, enabling selective delivery of highly potent cytotoxic agents to tumour cells while often maintaining their immunostimulatory properties. The classical construction of such conjugate is illustrated in figure 1.1. It consists of a targeting antibody, a linker and a highly cytotoxic agent often called the payload or warhead. Nine different conjugates are currently on the market, with an increasing number (S7) in the pipeline (1). But why and how were ADCs developed and what is their advantage over classic antineoplastic agents?

Figure 1.1: The classical construction of an antibody-drug conjugate with brentuximab vedotin as an example (2).

1.1 SITUATING A FIELD AND ITS PRACTICES

1.1.1 History and aspired advantages over classical molecules

In the battle against cancer, the immune system will typically generate specific antibodies with an antigen binding fragment directed towards epitopes of distinctive molecules on the plasma membrane of a tumour cell (3). These antibodies have some level of anticancer activity through natural mechanisms: complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent cellular cytotoxicity (ADCC) (4). Unfortunately, patients have often undergone previous therapies which have suppressed the immune system. Therefore, linking a cytotoxic drug to the antibody seemed like the perfect solution and was first described by the ‘founder of chemotherapy’ Paul Ehrlich in the early 1900s as a ‘magic bullet’ (5). The main objective for conjugation to antibodies was to improve the therapeutic index of the chemotherapeutics by delivering these specifically to cells expressing the target antigen of the selected monoclonal antibody (mAb). The concept of the German physician-scientist first came to life in the 1950s when methotrexate was linked with polyclonal rodent immunoglobulins through a diazo reaction (6). However, it wasn’t until the early 1970s when monoclonal antibodies with improved homogeneity and targeting accuracy could be produced using new hybridoma technologies, that the first ADCs gave promising results in vitro and in animal cancer models (7).
1.1.1  Selective targeting, higher concentrations of cytotoxic agents?

A well-known obstacle with classical chemotherapeutics is the minimal difference in effective and toxic concentrations. It was anticipated that this typical narrow therapeutic window could be improved by linking these drugs to specific antibodies that would allow to expose target cells to higher concentrations of the chemotherapeutic agent than other cells. The first clinical trials of ADCs began in the 1980s, but results were disappointing showing reduced effectiveness and similar toxicity assumed to be caused by poor target selection and the use of more immunogenic chimeric or murine antibodies. The reduced effectiveness was explained by the size of the ADCs, which complicates intracellular delivery as compared to conventional small cytotoxic drugs. In fact, data indicated that only a small fraction of the injected ADC molecules (in the order of 0.1%) made it to the intended tumour site. Rate of delivery and penetration are limited by characteristics depending on the antibody and therefore hardly adaptable (3,7,8).

1.1.2  Use of highly potent anticancer agents resulting in first generation ADCs

Because of the relatively low amount of drug that reaches the target site, highly cytotoxic drugs were selected for antibody conjugation, which critically increases the risk of severe toxicity when the agent is released during circulation. Ideally, the ADC should remain intact until it reaches the target cell. (3) In 2000 the first ADC, Gemtuzumab ozogamicin, was approved by the FDA for the treatment of relapsed and/or refractory (R/R) acute myeloid leukaemia. Its mAb targets CD33 and is linked to a calicheamicin, which causes DNA cleavage. After 10 years on the US market, the conjugate was voluntarily withdrawn by concerns of a negative risk/benefit balance only to be approved again in 2017 by the FDA as well as the EMA following reassuring high-quality data (9,10).

1.1.3  Second generation of ADCs

The brief success of the first approved ADC paved the way for a new, second generation of ADCs. Improvements were made by employing a more stable linker, a higher drug-to-antibody ratio (DAR) (see section 5) and a lower concentration of naked antibodies in the formulation. This strategy bore fruit and resulted in the approval of two additional ADCs, brentuximab vedotin in 2011 and trastuzumab emtansine (T-DM1) in 2013. However, their linker stability proved to be inadequate owing to decomposition of the thiol-maleimide moiety when reaching the blood circulation leading to off-target, off-tumour toxicity (see section 9.1.2). This issue with cysteine conjugated maleimide residues has been demonstrated in several pharmacokinetic studies. As a result, the enhanced therapeutic index that was envisioned with such a “magic bullet” was not fully achieved yet (2).

1.1.4  Most recent ADCs

The antibody-drug conjugates being investigated today aim for the broad therapeutic index that was not accomplished before. This generation is more homogeneous, potent and stable.
1.1.2 The answer to treatment-refractory cancers

Today, ADCs have succeeded in obtaining approval for a number of diverse indications, but their broader use is still a long way off. First, there will be some challenges to overcome, such as poor predictive biomarkers, their incomprehensible toxicity profile and drug resistance pathways as well as their unknown value when used together with classical therapies. However, many ADCs have proven useful for the treatment-refractory cancers known to be unresponsive to first-line therapy. In fact, all conjugates approved for solid tumours are indicated specifically for treatment-refractory tumours. Such cancers are predominantly heavily pre-treated and therefore often characterized by genomic instability causing inter- and intratumour heterogeneity, a hypoxic environment and immunosuppression. This is currently combated with palliative chemotherapy, but inevitable systemic exposure does not permit adequate dosing. Antibody-drug conjugates are able to deliver highly potent payloads with high selectivity to tumour tissues, creating a more suitable therapy for these cancers. These payloads are overall broadly cytotoxic, and the conjugates often generate immune-stimulatory effects. Moreover, the hypoxic environment may promote linker cleavage and therefore payload release, while the “bystander effect” may be beneficial with regard to tumour heterogeneity. This involves diffusion of the hydrophobic payload from the target cell (after internalisation) into adjacent cells (including, antigen-negative cells) (see section 8.2.2) (7).

1.1.3 A brief introduction to the mechanism of action of ADCs

An antibody-drug conjugate is administered via intravenous injection to prevent degradation by gastric acid or proteolytic enzymes. In other words, the conjugate is released directly into the blood stream from where distribution to the tumour tissues can begin. In practise only up to 0.1% will reach the desired area enabling target recognition and binding. From there on the ADC-antigen complex is internalized mainly through antigen-dependent endocytosis after which the payload gets released throughout the endosomal and/or lysosomal pathway. Dependent on the nature of the payload it may than affect different cellular targets such as microtubulins or DNA, thereby inducing apoptosis and ultimately cell death (11). Furthermore, it is anticipated that the efficacy of an ADC is determined by both antibody, linker and payload dependent factors, all of those being a function of complicated interactions among the ADC and numerous components of the tumour as well as the tumour microenvironment (TME). For example, the immunoglobulin backbone can often still exert its immunological effect (7). The mechanism of action (MOA) is further explained in section 8.
2 OBJECTIVES

The goal of this systematic review is to give a state of the art of antibody-drug conjugates while painting a picture of the challenges encountered with ADC design and how these were/are tackled. There will be an in-depth summary of the antibody backbone, linker and payload in the ADC construction and we will look into their mechanism of action and toxicity profile. Furthermore, this thesis will give an overview of the currently approved conjugates and will briefly discuss possible application beyond oncology diseases.
METHODS

Records identified through database searching (n = 886)

Additional records identified through other sources (n = 34)

Records after duplicates removed (n = 920)

Records screened (n = 920)  Records excluded (n = 704)

Full-text articles assessed for eligibility (n = 216)

Full-text articles excluded, with reasons (n = 116):
- A similar article was chosen because it was more recent.
- A similar article was chosen because of a higher journal impact and/or citation number.
- The article discussed a topic too detailed for this thesis.
- The article discussed a topic already mentioned in a general review.

Studies included in qualitative synthesis (n = 100)

Figure 3.1: PRISMA 2009 flow diagram.
Using the web of science database, a literature search was performed with the search term “antibody-drug conjugates”. The 886 articles of the past five years (2017-2021) were quickly reviewed by reading the title and abstract. Often, the topic was not about ADCs, or it was already clear at the time that the topic did not fit into the thesis. It was decided in advance to focus on the aspired advantages of ADCs over classical chemotherapeutics, an overview of approved ADCs, the development of ADCs with their most common hurdles, their mechanism of action and non-oncology indications. As a result, a total of 704 articles were not selected. Prior to the following selection, two general reviews on ADCs were thoroughly studied for better background knowledge: “Unlocking the potential of antibody-drug conjugates for cancer treatment” and “Strategies and challenges for the next-generation of antibody-drug conjugates” (2,7). The former was a recent review from 2021 published in a highly cited journal, whereas the latter was an older review from 2017 but with an astonishing citation number of 757. Third, the other 216 articles were explored, with a now improved knowledge of ADCs. They were grouped by topic, making it easy to select by publication date, journal impact and citation number. Furthermore, in this second selection it sometimes became clear that the article was too detailed for this thesis or that the subject was already described in a general review. This resulted in a final selection of 66 articles. An additional amount of 33 articles were used in this thesis. Occasionally, I would receive interesting articles from my supervisor or the answer to a specific question was searched for. But most of the time it concerns references from a previously selected article, in order to be able to give a more detailed description. Thus, in conclusion, a total amount of 100 articles was used in this thesis.
4 ANTIBODY BACKBONE AND TARGET ANTIGENS

4.1 ANTIBODY BACKBONE: ONE SIZE DOES NOT FIT ALL

Immunoglobulin G (IgG) (figure 4.1) is the most abundant backbone used, not only in ADCs, but also in conventional therapeutic antibodies (4). To lower the risk of an immune response in the form of hypersensitivity or neutralising anti-drug antibodies one mainly works with humanized or chimeric immunoglobulins. However, it’s important to note that this does not entirely preclude these risks. Human IgG includes four subclasses which differ structurally in their constant domains and hinge regions, but also characteristically in their solubility and half-life properties, their complement fixation and affinity for different Fragment crystallizable γ receptors (FcγR) on immune effector cells. As listed in table 4.1, IgG1 is most commonly used because of superior ADCC and CDC abilities resulting in a more immunogenic subclass compared to IgG2 and IgG4 (7). Per contra, limited effector functions are occasionally preferred if it is assumed that ADCC plus the payload will be too toxic, in particular when the expression and cellular distribution of the target antigen are unfavourable (2,12). Furthermore, IgG1 enjoys the benefit of a relatively easy development as IgG2 contains a complex hinge region. Likewise, IgG4 may fall apart resulting in two times one heavy and one light chain or form bispecific antibodies after entering the body unless the hinge region is stabilized through mutation (13). It is also harder to reduce the disulphide bridges present in the hinge regions of IgG2 and IgG4. IgG3 antibodies have been avoided altogether in ADC design and antibody-based therapeutics due to their relatively short circulating half-lives (2).

In conclusion, selection of an antibody backbone will depend on the tumour type and the expression pattern. For example, when the target antigen exclusively occurs on the tumour cells, as is often the case with liquid tumours, IgG1 would be the best option. If not, IgG2 or IgG4 backbones should be considered (12). Of note, monoclonal antibodies optimized for other applications than ADCs may not be best option. This is because internalization and intracellular trafficking of the conjugate also influence ADC activity (7). For the ADC to accumulate into the tumour cells, the rate of receptor-dependent internalisation should beat the receptor-independent endocytosis.
Furthermore, it has been reported that drug conjugation may influence the internalisation rate, although rarely. One study suggests that target antigen affinity correlates with internalisation rate (4).

Table 4.1: Immunoglobulins G subclasses (7,14).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Serum half-life</th>
<th>C1q binding (CDC)</th>
<th>FcγR affinity (ADCC/ADCP)</th>
<th>X/9 used in approved ADCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>21 days</td>
<td>Yes</td>
<td>High</td>
<td>7/9</td>
</tr>
<tr>
<td>IgG2</td>
<td>21 days</td>
<td>Yes</td>
<td>Low</td>
<td>0/9</td>
</tr>
<tr>
<td>IgG3</td>
<td>7-21 days</td>
<td>Yes</td>
<td>High</td>
<td>0/9</td>
</tr>
<tr>
<td>IgG4</td>
<td>21 days</td>
<td>No</td>
<td>Moderate</td>
<td>2/9</td>
</tr>
</tbody>
</table>

4.1.1 Bispecific antibody-drug conjugates (bsADC)

Bispecific antibody-drug conjugates are artificially designed immunoglobulins enabling simultaneous binding of two distinct target antigens. bsADCs are being developed in the hope of increasing tumour selectivity and safety or to enhance internalization/degradation benefitting efficacy (15). Proof of concept was reported for the additional targeting of high turnover proteins with bispecific antibodies. HER2, an oncogenic target of two approved ADCs (see section 9), is overexpressed in various tumour types including breast cancer. However, it is believed that internalised HER2 undergoes extensive recycling where the endosome is transported back to the cell surface. In contrast, prolactin receptor (PRLR), an oncogenic protein with low expression in a subset of breast tumours, undergoes rapid and constitutive internalization followed by efficient trafficking and degradation. Because efficient intracellular trafficking can significantly boost efficacy these two cell surface proteins were crosslinked by usage of a bispecific antibody (figure 4.2). Promising results were obtained with elevated degradation of HER2 and more effective cell killing of the bsADC compared to the HER2-targeted ADC. Moreover, a noncompeting HER2-targeting ADC also exhibits improved activity when combined with a HER2xPRLR bsADC (16). Biparatopic antibodies in particular, which can recognize two unique epitopes on the same target antigen, are thought to enhance receptor clustering and accelerate internalization. MEDI4276 is such a biparatopic ADC exerting accelerated internalization and trafficking after induced HER2 clustering. This translated into improved activity both in vitro and in vivo compared to trastuzumab emtansine (17).

On-target, off-tumour toxicity (see section 9.1.2) may pose safety problems for bsADCs. Thus, complementary targets should be carefully selected with regard to individual antigen binding fragment (Fab) affinity and target density (15). Domination of one antigen binding fragment is possible. Nevertheless, bispecific antibodies are a promising extension of ADCs currently under investigation (7).
Figure 4.2: Mechanism of action of a HER2xPRLR bsADC enhancing endosomal/lysosomal trafficking and degradation leading to higher efficacy in comparison with a HER2-targeting ADC [16].

4.1.2 Small format-drug conjugates

It is known that tumour penetration is limited by the magnitude of the antibody backbone, stimulating the search for smaller conjugates. Especially low vascularized tumours or the central nervous system could benefit from smaller ADCs [7]. Small ADCs might undergo accelerated extravasation and diffusion leading to improved penetration [18] and a better, more homogeneous distribution [19]. However, conventional immunoglobulin backbones still achieve a higher absolute concentration at the tumour site owing to their longer half-lives. This can be explained by the size-dependent permeability of the glomerular membrane (threshold 60-70 kDa) and the salvage recycling (neonatal Fc receptor pathway, see section 8.1.2.1). Furthermore, because small ADCs have no Fc domain, there is less cross-reactivity with Fc-receptors on normal cells leading to a better toxicity profile. As an additional advantage small ADCs undergo less off-target payload release due to a generally lower plasma concentration. Of note, the pharmacokinetics of small conjugates will be more influenced by the characteristics of the payload, which are a more important part of the construct. To reduce the size of an ADC one may use antibody fragments, scaffold proteins, peptide fragments, or small molecules. A more detailed overview of all small conjugates is given in reference [18].

Antibody fragments (see figure 4.3, A) are a first, obvious strategy in developing smaller conjugates including Fab, single chain variable fragments (scFv) and non-covalent dimers of these single-chain variable fragments, also called diabodies (bsADCs). scFvs consists of a heavy and light chain of the variable region linked by a peptide sequence or/and a disulphide bridge. Considering scFvs are relatively small (see figure 4.3, A (e)) tumour penetration is relatively high. In addition, it was discovered that exceptionally high DARs (8-10) could be achieved using lysine conjugation. Lower plasma clearance was observed due to the high conjugation rate of hydrophobic payloads, which inhibited renal clearance and led to a redistribution to the liver. This method, explored by the company Antikor, showed great in vitro potency in the picomolar range when conjugated to MMAE with minimal side effects on normal cells [18,20]. Antibody fragment-drug conjugates also include
nanobodies, the smallest naturally derived antigen-binding fragment (15 kDa) comprising the variable heavy chain domain of antibodies first discovered in camels and llamas and extensively explored as therapeutics by Ablynx. Nanobody-drug conjugates are currently being investigated (21). A second method comprises smaller engineered proteins, mimicking antibody fragments, termed scaffold proteins (6-21 kDa) (figure 4.3, B). For example, affibodies based on the Z-domain of protein A found in Staphylococcus. These were designed more than 20 years ago, but mainly as imaging or anti-inflammatory purposes. Interest in these molecules as targeting ligands is based on their relatively straightforward production, characterisation, thermostability and high affinity compared to antibodies. Mainly HER2 targeting affibodies are evaluated in vitro, but further optimisation is needed to proceed to in vivo studies, e.g. to tackle their short serum half-life of 20 minutes (18,22). Another strategy to develop small-format drug conjugates are peptides molecules. Peptides benefit from a relatively cheap, convenient and large-scale synthesis. They exhibit even faster tumour penetration and clearances rates than the variations described above (18). For example, a 2kDa peptide-DM1 conjugate is currently under investigation (NCT02936323). Moreover, in 2014, a small peptide linked to three paclitaxel compounds (paclitaxel trevatide) against glioblastoma was launched on the market through an FDA orphan drug designation (7). Glioblastoma is an extremely aggressive brain tumour. Therefore, angiopep-2 was selected as the peptide backbone since it can cross the blood-brain barrier through low-density lipoprotein receptor-related protein 1 (LRP1) mediated transcytosis. Because LRP1 is also found on the surface of tumour cells, the peptide-drug conjugate can enter through endocytosis (23). A fourth category consists of small molecules (around 0.2 kDa) as the targeted ligands. For example, acetazolamide (AAZ), a ligand for the tumour associated enzyme carbonic anhydrase IX (CA-IX). The payload is extracellularly released considering that CA-IX does not internalize efficiently. A comparative evaluation of a high affinity AAZ-derived small molecule-drug conjugate (SMDC) (DAR 1) and a corresponding ADC (DAR 2) showed superior ADC efficacy for the latter. However, the SMDC exhibited great specificity, high tumour uptake and penetration. It was concluded that efficacy could be benefitted by accelerated payload release appropriate to SMDC pharmacokinetics (18,24).

As mentioned before, these small conjugates are characterized by rapid plasma clearance, complicating their therapeutic potential. Luckily there are two emerging technologies to solve this issue. By linking an albumin-binding domain (ABD) to the backbone, the conjugate is able to interact with albumin, the most abundant protein in plasma. This molecule can be recycled through the neonatal Fc receptor pathway and can’t pass the glomerulus due to its size. Additionally, albumin can bind to SPARC (secreted protein acidic and rich in cysteine) making extravasation through the gp60 signalling pathway possible, ultimately leading to improved accumulation in solid tumours. Alternatively, PEGylation can also increase the hydrodynamic radius above the renal threshold. Moreover, the magnitude and the form of the polyethylene glycol (PEG) has a significant impact
on distribution and tumour uptake. A study that examined these technologies successfully concluded that ABD linkage and PEGylation could prolong the half-life of diabodies from minutes to days (19).

![Image](image1.png)

Figure 4.3: Illustration of various antibody-fragments (A) and scaffold (B) targeting ligands (18).

4.1.3 Chemical engineering of antibody backbone to elevate internalization

Cell-penetrating peptides (CPPs) are short sequences of maximum 30 amino acids known to easily cross the cell membrane. These molecules were used before in the context of molecular imaging with antibodies but were not explored with regard to ADCs. A proof-of-concept study investigated the effects of conjugating a tetrameric cell-penetrating peptide (tCPPs) to mAbs and to an ADC. The choice of a multimeric peptide came after the report that the amount and not the size of modification led to lower target specificity. Thereby, it was hypothesized that multimerization led to an additive effect. A promising increase in internalisation rates was observed, while cellular binding was hardly affected. Also, conjugation also led to improved pharmacokinetics and less off-target accumulation. Additionally, it is expected that tCPP-mAb conjugates may broaden the target scope since tCPPS can also induce internalisation of antibodies targeting slowly or non-internalizing antigens due to an extra internalisation apart from the targets intrinsic one. In vitro evaluation of the immune response to these conjugates showed no higher levels of cytokines (25).

![Image](image2.png)

Figure 4.4: Visualisation of a tCPP-mAb-conjugate.
4.2 TARGET ANTIGEN SELECTION

In the past, target antigens were selected based on three main criteria. Although these are still relevant today, one now follows an integrated approach taking into account all biophysical characteristics of the final conjugate and the clinical development strategy. This is related to the expansion of ADC platform technologies, the increased knowledge in tumour biology and the obtained clinical data on ADCs. A first important and logical strategy for selecting a suitable target antigen is to look for cell-surface proteins that only (tumour-specific) or prevalently (tumour-associated) occur in malignant cells. If interaction with normal cells is possible then there might be implications for both safety and efficacy as ADC exposure at the tumour action site will diminish. Additionally, absolute (next to relative) target antigen expression may also be important, as shown for the microtubule targeting agents (26). Indeed, the threshold level of tumour-specific target expression required for ADC activity will depend on the degree of differential expression in malignant and non-malignant cells (7). Especially the linker and payload choice can impact the level of expression required for efficacy/toxicity. For example, efficacy of an ADC against target antigens with low expression levels can be increased by compensating with a more potent payload. (26). Second, it was previously assumed that the target antigen, in order for the conjugate to display toxicity, had to internalize leading to intracellular release of the antibody and payload (2). A fast internalization rate is still preferred as it prevents target antigen-independent endocytosis, but non-internalizing target antigens have also proven efficacious due to a strong bystander effect (see section 8.2.2). Non-internalizing antigens expand the scope of potential mAb candidates because they are independent from intracellular trafficking and do not require high cell-surface expression levels (2,4). Again, the linker is preferably selected with the internalization and trafficking of the antigen in mind. Moreover, it might be possible to define novel targets based on difference in internalization abilities between normal antigen-positive cells and malignant cells. The third condition calls for the target antigen to be present on the surface of the cell to be accessible for the ADC, but many other factors may play a role as explained below (26). The fact that the target antigen does not have to be a part of tumorigenesis because the primary mechanism of action is performed through the payload is considered a major advantage of ADCs. However, if the selected target antigen plays a functional role in the mechanism of the cancer, the corresponding ADC might be less prone to drug resistance caused by downregulation of the target. Because downregulation would also result in a drawback for the tumour itself (26). Moreover, it can contribute to a greater ADC activity through antibody-mediated suppression of downstream oncogenic signalling pathways (7). Nevertheless, therapeutic resistance with trastuzumab emtansine, an approved ADC targeting an oncogenic driver, has been reported (26). Mutant tumour proteins may be valuable target antigens as they often undergo higher ubiquitylation, internalization and/or turnover as opposed to their non-mutant forms. Even more, targeting truncal oncogenic driver mutations like some variants...
on EGFR may increase tumour specificity to levels only accomplished by small-molecule tyrosine kinase inhibitors (7). An ADCs can also be directed towards stromal or vascular target antigens in the TME, considering this environment encourages tumour cell growth. The conjugate can exert its effect by degeneration of the TME and/or degeneration of the tumour cell through the bystander effect. Logically, non-internalizing antigens are not desirable when endothelial cells are targeted to prevent payload release into the blood circulation. These targets impose an additional benefit since they are often generalizable among various tumour types (27). Genomics and proteomics can replace mRNA profiling in the search for new target antigen candidates, such as tumour specific protein variants formed through distinctive splicing or post-translational modifications while keeping in mind the absolute expression levels. Additionally, multiple binding sites on the target antigen can enhance conjugate binding and therefore compensate for insufficient absolute expression. Of note, enhanced binding will probably occur on normal cells as well. Antigen shedding has also to be taken into account as it results in soluble and circulating target antigen leading to toxicity (26). Furthermore, the homogeneity of target expression at the level of tumour type (intratumour) as well as at the level of the patient (intertumour) is critical. Intratumour heterogeneity, meaning a higher percentage of target antigen-negative cells, imposes worse clinical outcome. Intertumour heterogeneity causes boundaries on patient population (28). Lastly, rapid target antigen turnover might stimulate drug delivery, while target replenishment and lysosomal trafficking might also impact drug release. In conclusion, integral approach enables selection of next-generation targets through consideration of the antibody, drug, bioconjugation, linker and clinical development strategy (26). Of note, haematological or liquid tumours are easier to treat than solid tumours because of lineage-specific target antigens, homogeneous expression, low level of expression in normal solid tissues, relative high drug exposure in blood tumour tissue compared to solid organs and no need for penetration into the solid tumour to exert its effect. Lastly, the fast and continues turnover due to the hematopoietic stem cells can compensate for low relative expression. Thus, even when the ADC additionally attacks normal blood cells, they will be quickly replenished by the hematopoietic stem cells (HSCs) (26).

4.2.1 Innovation
Identification of adequate and very specific target antigens is crucial for further refinement of ADC development. Intracellular proteins presented by human leukocyte antigens (HLAs) on the surface of tumour cells or artificial receptors meet this requirement. Artificial receptors can be generated on the cell surface through usage of characteristics of the tumour cell and TME (29,30). Additionally, certain proteins or peptides have been developed to mask the antigen-binding site of the conjugate, only to be released by exposure to the TME (31). Two of these ADCs, often termed probody drug conjugates, are currently in phase 2 clinical trials.
5 CONJUGATION STRATEGIES

Conjugation is one more critical element in the development of ADCs. Therefore, site-specific coupling strategies were developed during the last decade to supersede conventional methods like cysteine or lysine conjugation. An important concept in this context is the average number of payload molecules attached to a single mAb, called the drug-to-antibody ratio (DAR). This DAR has an impact on the ADC’s pharmacology and activity, for instance, higher DARs are expected to be more potent in vitro. However, data from preclinical studies suggest that the overall hydrophobicity of the conjugate will increase with the number of cytotoxic agents linked to the mAb. This might explain the faster liver clearance rates of some of these higher DARs leading to comparable in vivo activity as with lower DARs. Usage of hydrophilic constructs can counteract this relationship and for some ADCs like sacituzumab govitcan drug conjugation does not have an impact on plasma clearance. In such cases conjugates with higher DARs can lead to better results in vivo (7). Physicochemical features of the conjugation site such as solvent accessibility and local charge might influence both pharmacokinetics and -dynamics (32). Thus, a well-defined, consistent DAR and site of conjugation is crucial if only to avoid a complex mixture with both naked and overloaded antibodies. Naked antibodies can act as competitive inhibitors whereas overloaded conjugates may cause aggregation and an overall pharmacological disadvantage. Additionally, analytical characterisation and purification becomes more challenging in the case of inconsistent conjugation (33,34).

5.1 LYSINE AND CYSTEINE CONJUGATION

The antibody backbone is a protein macromolecule composed of amino acid residues, some with nucleophilic side chains suitable for conjugation. In fact, all currently approved ADCs utilize pre-existing lysines or cysteines (33). Lysine has a nucleophilic primary amino group which can be acylated or alkylated by a number of drug linkers, e.g. bearing an activated carboxylic acid ester (3,35). For example, amide-coupling of an amine residue is realized through treatment with an N-hydroxysuccinimidyl (NHS) ester of a carboxylic acid containing drug linker (figure 5.1) (35,36). In ado-trastuzumab emtansine (see 9.3), NHS coupled with a non-cleavable linker bearing a maleimide is utilized for conjugation. A maleimide can ligate the linker to any drug with a nucleophilic sulfhydryl moiety (36). There are several downsides to this heterogeneous approach: lysine-coupling may occur in the region responsible for interacting with the target antigen, uncontrolled lysine coupling resulting in DAR variation and precipitation of the antibody-drug conjugate due to masking of polar amino groups (3,7). Moreover, it has been calculated that over one million different ADC variants can be formed through lysine conjugation (37).

Figure 5.1: Lysine amide coupling between a amine and a activated carboxylic acid ester (NHS) (35).
Through cysteine conjugation, coupling can be carried out in a more controlled fashion while the antigen recognition site is not altered. The hinge region of the antibody contains four interstrand disulphide bridges which can be reduced to eight thiol groups to attach the same number of drug molecules through alkylation or via a disulphide linkage (3). A thiosuccinimide linkage obtained by reaction of a thiol with an alkyl maleimide (figure 5.2) is most common considering its rapid, selective and quantitative reaction (2). These interstrand disulphide bridges have no crucial impact on the backbone structure and are more sensitive to reduction compared to their intrastrand variants (32). Thereby the heterogeneity issue was partly resolved as this strategy generates “only” around 100 different ADC variants (37). To further address this problem, “cysteine rebridging” was designed. This boils down to bis-alkylation conjugation at reduced interchain disulfides (figure 5.2) producing homogeneous conjugates without use of antibody engineering techniques. Along with structure and DAR stability this could possibly lead to a better pharmacokinetic/pharmacodynamic (PK/PD) profile (25). A general application of this method has been reported in 2019 (38).

Figure 5.2: Example of cysteine conjugation using a maleimide linker (A) and cysteine rebridging (B) (35).

5.1.1 Counteract retro-michael deconjugation

As mentioned, maleimide structures are convenient to ligate thiol containing drug molecules to a nitrogen of the antibody. This Michael addition is rapid, selective and quantitative and therefore widely used. However, it turned out that this conjugation was reversible in plasma leading to premature drug release and loss of efficacy (figure 5.3). Thiol exchange with human serum albumin is possible. Several solutions were devised such as self-hydrolysing maleimide structures consisting of a basic amino group to cause hydrolysis of the ring (figure 5.3). The structure is thereby stabilized and resistant to deconjugation (39,40).

Figure 5.3: visualisation of a retro-michael deconjugation (A) and a self-hydrolyzing maleimide (B) (39,40).
5.2 SITE-SPECIFIC CONJUGATION FOR HOMOGENEOUS, NEXT-GENERATION ADCs

Several strategies for homogeneous ADCs have been developed in recent years but turning these into robust and scalable techniques remains a challenge since none have so far afforded approved conjugates (41).

5.2.1 Engineered cysteines

Engineered cysteines can be introduced into various sites (with different local charges or solvent accessibility) of the antibody. Identification of fitting incorporation sites is key to successful conjugation as antibody structure and function need to be preserved upon generation of THIOMABs backbones and ultimately TDCs (32). This was the first approved technology enabling not only uniform stoichiometry (with DARs near 2 or 4), but also site-specific conjugation (2,37). The engineered cysteine residues are initially ‘blocked’ with cysteine or glutathione. Following reduction of all disulphide bonds and spontaneous reoxidation of interchain cysteines not affecting the engineered cysteine residues, the ‘deblocked’ THIOMAB is ready for conjugation with a thiol-reactive linker (figure 5.4) (42). Other cysteine engineered antibodies have also been developed in addition to THIOMABs. Of note, it was reported that TDCs are prone to early payload release due to maleimide exchange with other reactive thiols present in plasma constituents. However, conjugation to a partially accessible, positively charged site causes hydrolysis of the succinimide ring in the linker reducing the maleimide exchange reaction (2).

![Figure 5.4: Site-selective conjugation through engineered cysteine technology (42).](image)

5.2.2 Unnatural amino acids (UAAs)

The genetic code is known to translate 20 standard amino acids but through biotechnological advances it has been made possible to encode unnatural amino acids (UAAs) with bioorthogonal chemical reactivity (32). A bioorthogonal chemical reaction can occur inside a living organism without interference of the native biochemical processes (2). This requires an orthogonal tRNA/aminoacyl-tRNA synthetase pair that inserts an UAA at a specific site in the growing polypeptide (the antibody) as a result of a stop codon substituted for a mRNA coding sequence in the gene of interest. For example, an UAG stop codon was incorporated in an IgG gene while IgG was co-expressed with an orthogonal UAG tRNA/aminoacyl-tRNA synthetase pair in a mammalian cell line. The aminoacyl in particular, para-acetylphenylalanine (pAcPhe), possesses a carbonyl group allowing to specifically couple an alkoxylamine linker drug. This chemical reaction, optimized for the conjugation of auristatins (DAR 2), has proven to be efficient, scalable and to result in a very stable linker (2,43).
In a similar manner, azide antibodies were produced by incorporation of UAAs with an azide handle. This allowed copper(I)-catalysed azide-alkyne cycloaddition (CuAAC) or strain-promoted azide–alkyne cycloaddition (SPAAC) reactions, implicating swift conjugation with an alkyn group to form a stable triazole (2). SPAAC is the preferred strategy since use of the cytotoxic, oxidative copper catalyst is not necessary with a strained cyclooctyne linker (35). Using this highly selective conjugation reaction, the payloads monomethyl auristatin F (MMAF) and pyrrolobenzodiazepine (PBD) could be coupled to an aromatically stabilized linker giving a toxicity advantage over maleimide or thioester strategies (2). Furthermore, a cell-free expression system has been developed enabling incorporation of the UAA para-azidomethyl-l-phenylalanine (pAMF). Subsequently, near complete conjugation with DBCO-PEG-MMAF through SPAAC is possible. This in vitro transcription/translation approach enables a rapid, cost-effective, virus-free and scalable manufacturing (43). Incorporation of selenocysteines, which is more nucleophilic than cysteine itself, is another option. The reoxidation step, as with THIOMABs, is then no longer required (43).

UAA-conjugation is advantageous over engineered cysteine or peptide tags strategies since maleimide exchange in plasma or the additional enzymatic processing steps can be avoided (44). Additionally, a consistent DAR can be assured by adapting the load of UAAs while fully using their reaction handles (35). Identification of fitting conjugation sites is, again, crucial and evaluation of immunological reaction is required (34,35). Although rigorous and efficient conjugation is now possible, further methodology improvement for industrial production is required. In 2020 a process to select stable cell lines with efficient UAA incorporation and higher yields was reported (45). The conjugation methods discussed are illustrated in figure 5.5 (35).

![Figure 5.5: Visualisation of oxime ligation (A), copper catalysed reaction (CuAAC) (B) and strain-promoted azide-alkyne cycloaddition (SPAAC) (C) following non-natural amino acid incorporation (35).](image-url)
5.2.3 Glycan-mediated conjugation

As the IgG backbone is N-glycosylated at the conserved asparagine 297 (N297) located in the CH2 domain, this is seen as a unique, specific conjugation site distant from the antigen-binding region. Numerous bioconjugation strategies have been designed such as metabolic engineering, chemical oxidation and (chemo-)enzymatic modification. An unnatural fucose derivative like 6-thiofucose can be metabolically incorporated when added to the culture media. Subsequent improved conjugation through a maleimide drug-linker is now possible. Chemical glycan modification takes place through oxidation using NaIO₄. The vicinal diols of the carbohydrates including galactose or sialic acid are thereby converted to aldehyde groups ready for conjugation with a hydrazone linker drug (2). Furthermore, there are several (chemo-)enzymatic approaches. For example, both N-glycan chains can be shortened by endoglycosidase Endo S2, followed by N-azidoacetyl- galactosamine (GalNAz) extension using a mutant galactosyl transferase GalT(Y289L). The azide group enables SPAAC conjugation (figure 5.6). Because of its improved stability and homogeneity, this so-called GlycoConnect technology is gaining increasing interest. Furthermore, any IgG isotype, regardless of N-glycosylation profile, can be exploited using this approach (35).

Figure 5.6: Illustration of the GlycoConnect technology (35).

5.2.4 Short peptide tags for enzymatic conjugation

Another conjugation strategy relies on the site-specific incorporation of short peptide sequences in the antibody backbone (2). These peptide ‘tags’, comprised of four to six amino acid residues, are in turn recognized and functionalized by certain enzymes. One such enzyme, formylglycine-generating enzyme (FGE), oxidizes the cysteine residue in the peptide tag (Cys-X-Pro-X-Arg) to formylglycine, which bears an aldehyde handle that can be efficiently conjugated to an aminooxy or hydrazide drug linker (2,43). A second enzyme utilized is microbial transglutaminase (mTGase), which catalyses a transpeptidation. mTGase has been engineered to specifically recognize a peptide tag and may be used to attach an ADC linker possessing a primary amine to a glutamine residue. But this enzyme is also exploited without an incorporated amino acid sequence, in this industrially applicable method, two enzymes are employed to simultaneously modify and to conjugate the antibody. A glycosidase is used to remove the glycan bound on the asparagine residue N297. Then, the nearby glutamine residue Q295 can be specifically coupled with an amine drug linker by an mTGase. This results in an
ADC with a DAR of two (one per heavy chain), but when using a mutant (N297Q) IgG1 backbone, the DAR can be raised to four (32,35). Recent advances regarding mTG conjugation include further optimization of the mTGase and recognition motifs (46,47). Additionally, mTGase was engineered enabling conjugation to native, glycosylated IgG backbones. As described, most site-specific methods demand alternation of the antibody structure or its glycosylation profile (48). Likewise, sortase A can be applied for conjugation as it will break the threonine-glycine bond in a Lys-Pro-X-Thr-Gly sequence and attach an oligoglycine drug linker. Numerous drug linkers can be attached to an oligoglycine. This peptide tag can be engineered into terminal sites of the heavy and light chains facilitating the transpeptidation reaction (35).

5.2.5 Affinity peptides
AJICAP is a new technology for chemical specific conjugation of intact native antibodies. Briefly, Fc-affinity peptides were identified, able to conjugate drug compatible linkers to specific lysine residues. Redox treatment will then lead to cleavage of these linkers revealing a site-specific thiol modified antibody. Various maleimide drug linkers can now be coupled to the antibody (figure 5.7). This technology has evolved in a reproducible and gram-scale synthesis. A good manufacturing practise has been developed for future industrial use (41,49).

![Figure 5.7: Illustration of the AJICAP technology (41).](image)

5.2.6 Innovation
Incorporation of two distinct payloads, has been hypothesized to result in more effective conjugates. For example, a dual, site-specific antibody conjugate has been constructed through one-pot chemoenzymatic ligation. The engineered antibody contained a peptide tag recognized by lipoate-acid ligase A and was ligated with an extracellular protease cleavable drug linker. A second, intracellular protease cleavable drug linker was conjugated to glutamine Q295 through mTGase. The resulting ADC enabled sequential release of two payloads, e.g. an efflux pump inhibitor and a hydrophobic drug (50). In 2021 a fast, versatile technology platform has been reported utilizing CRISPR/CAS9 engineered hybridoma cells for production of Fab’ fragments bearing two different recognition motifs. These backbones are ready for chemoenzymatic coupling with two unique drug linkers by two mutant sortase A variants. It is assumed that selection of two payloads with separate mechanisms of actions could partly prevent tumour resistance. Furthermore, this method enables rapid production and coupling of non-drug moieties like PEG chains for half-life extension (51).
6 LINKERS

Linkers are used to connect the antibody backbone to a payload. The various conditions that these linkers must fulfill are described below. First of all, it is crucial for a linker to remain intact until it has reached its target or TME. If not, premature release of the highly potent cytotoxic agent might be detrimental to healthy tissues. Additionally, the free payload would not reach the intended target cell. On the other hand, the conjugate should eventually release its toxic payload in order to kill the tumor (7,35) and to exhibit superior therapeutic properties than the naked antibody (7). A general optimal linker is non-existent. For example, a clinical study investigating a particular ADC showed a reverse correlation between linker stability and toxicity profiles (35). Reports from preclinical studies indicate that extracellular release of the payload might contribute to ADC activity. That is, in certain cases due to killing of nearby target antigen-negative cells (7). Thus, selection of linkers with a suitable stability is a complex matter and will depend on the target, the payload and the prevailing tumor microenvironment and its features. A third consideration is the hydrophobicity of the linker since this might trigger aggregation, certainly in combination with hydrophobic payloads. This leads to issues regarding immunogenicity, increased renal accumulation and clearance leading to hepatotoxicity (33).

6.1 CLEAVABLE LINKERS

Cleavable linkers are designed to decompose in reaction to the inherent properties of the tumor, enabling selective delivery of the payload (7). They are preferred due to their broad applicability but require extra vigilance with regard to tumor specificity. Linker stability also depends on the conjugation site since some regions on the antibody are more shielded from chemical or enzymatic triggers than others (52).

6.1.1 Chemically cleavable linkers

6.1.1.1 Acid cleavable linkers

Based on pH difference between plasma (7.4) and lysosomes (4.5-5.0)/endosomes (5.5-6.2), acid labile linkers were developed. N-acyl hydrazones hydrolyse into a hydrazide and keton-payload (figure 6.1) through acid catalysis and are used in gemtuzuzmab ozogamicin and inotuzumab ozogamicin (see section 9). In vivo results of inotuzumab ozogamicin showed good linker stability as only 1.5-2% was daily hydrolysed in plasma. However, the stability has proven critically dependent on the assay used, whereby for yet unknown reasons some hydrazones tend to be more prone to hydrolysis in plasma than in buffer. Considering their wide variety in stability profiles, interest in hydrazone linkers has decreased. Another acid labile moiety consists of a carbonate and a para-aminobenzyl (PAB) (figure 6.1) stabilizer and made it to phase I/II trials. In spite of great research efforts, various studies confirmed that efficacy observed for ADCs with cleavable linkers is partly due to extracellular release of the payload, followed by diffusion through the cell membrane. Therefore, use of moderately potent payloads (such as ozogamicin) seems appropriate. Additionally, linker discrimination
between pH 7.4 and S in vivo has turned out to be more then challenging and for these reasons, attention has
shifted to other possibilities (52).

Figure 6.1: Illustration of a hydrazone linker (A) and a carbonate-PAB linker (B) (35,52).

6.1.1.2 Reducible linkers

A reducible disulphide bond is most commonly used as chemically cleavable linker and found in gemtuzumab
ozogamicin and inotuzumab ozogamicin. These ADCs, both patented by Pfizer, bear the same linker comprising
both a reducible disulphide and a hydrazone moiety (52). This disulphide can be reduced by a nucleophilic thiol
such as glutathione (GSH), a tripeptide with high intracellular abundance (1-10 mmol/L) (35). This mechanism is
shown in figure 6.2. Often GSH concentrations are even higher in tumour tissue than in healthy cells due to
oxidative stress (52). In theory, this linker could also be degraded by human serum albumin, but this rarely occurs
because it is less abundant (5 μmol/L) and its thiol group has limited solvent exposure (35). Furthermore, the
disulphide linkage tends to remain intact at pH 7.4 (52).

Figure 6.2: Visualisation of the reduction of disulphide linkers (52).

To further improve linker stability in plasma, several studies experimented with steric hindrance around the
disulphide. It could be concluded that attachment of α-methyl groups can enhance stability, while medium steric
hindrance seems to demonstrate an optimal balance between plasma stability and cytosol cleavage efficiency.
Again, one size does not fit all and optimisation of a linker depends on the whole construct and tumour type.
Maytansinoids that contain a thiol group are often conjugated with this type of linker considering facile
conjugation. More recently, engineered cysteines were directly linked to maytansinoids, creating a linkerless ADC
(figure 6.3). Steric hindrance was delivered by the antibody and proved to be site-dependent while limited
solvent exposure favoured. α-Methyl substitution further enhanced stability leading to impressive antitumour
outcomes in mice. These results were contributed to a rise in plasma stability along with efficient cytosol
 cleavage. To further exploit this technology for more payloads, a self-immolating carbamate linker (figure 6.3)
was developed. This allows for cytotoxic agents like monomethyl auristatin E (MMAE) or PBD to be conjugated
through their secondary amine group. α-Methyl substitution provided not only more stability but also higher self-immolation. This straightforward linker type proved equipotent in vivo to a more complicated protease linker bearing the same payload (S2).

Figure 6.3: Cleavage mechanism of a linkerless cysteine ADC (A) and a self-immolating disulphide-carbamate linker (B), both releasing amine-linked drugs (S2).

These types of linkers can be utilized with non-internalizing antibodies as lysis of dead tumour cells together with low oxygen supply turns the TME to a reducing environment. Moreover, when the ADC starts to work, a chain reaction occurs due to increasing tumour cell death and an ever-reducing environment (S2).

6.1.3 Linkers cleaved by endogenous stimuli
This two-step technology, although complex, can benefit from the fact that linker cleavage is independent from endogenous trigger concentrations which varies across patients. For example, a thioether linker was designed that cleaves into an amine linked payload when exposed to Pd⁰. This linker was conjugated to a HER2+ targeting nanobody added to kidney cells together with [Pd(COD)Cl₂]. Results were positive, however, a less toxic Pd complex is needed for application in vivo (S2).

6.1.2 Enzyme cleavable linkers
Enzyme cleavable linkers enable selective intracellular release as the conventional mechanism of action includes lysosomal trafficking exposing the ADC to a high level of unique enzymes after internalisation. Extracellular release in the TME can also be selective if certain enzymes are predominant relative to healthy tissues (S2).

6.1.2.1 Protease cleavable linkers
Protease cleavable linkers were initially designed to target cathepsin B, a lysosomal cysteine protease overexpressed in a broad range of tumour types with an additional TME occurrence. Cathepsin B, with its carboxydipeptidase activity, hydrolyses the bond between a dipeptide and a drug attached to the c-terminal carboxylic acid. Structure–activity relationship (S.A.R) studies indicated that the amino acid bound to the drug must be hydrophilic and basic while the second amino acid is preferably hydrophobic (figure 6.4). This dipeptide is linked to the drug via a self-immolative para-aminobenzylcarbamate (PABC) spacer to combat steric hindrance. Through this popular method, MMAE and doxorubicin have been linked to an antibody, resulting in the
approved brentuximab vedotin bearing doxorubicin. A valine-citrulline combination proved to exhibit the highest in vivo activity (S2).

Figure 6.4: Possible combinations of a dipeptide linker along with its cleavage mechanism (S2).

Because preclinical studies in mice are of great importance and because this dipeptide-drug structure proved to be sensitive to an enzyme found in mice, a third hydrophilic and acidic peptide was added at the N-terminus without consequences for hydrolysis by cathepsin B. Glutamate-valine-citrulline appears to be the most suitable candidate based on various studies. Despite cathepsin B being the original target, several in vitro studies suggest that these valine-citrulline linkers are cleaved by multiple proteases. Nonetheless, cathepsin B is more abundant in cancer metastases than other proteases and therefore a cyclobutane-1,1-dicarboxamide (cBu) linker with higher selectivity for the enzyme was designed. The cBu linker was tested in mice xenografts linked to PBD and MMAE showing identical or improved results. Furthermore, valine-alanine dipeptide linkers bearing relatively lipophilic PBD dimers or other drug molecules suffered less from aggregation. This linker demonstrated sufficient protease hydrolysis and plasma stability (S2).

6.1.2.2 Glycosidase cleavable linkers

β-Glucuronidases are another type of lysosomal enzymes, catalysing the cleavage of the β-glucuronide-glycosidic bond (figure 6.5). These catalysts are only found in the lysosome or in some TMEs due to cell lysis. Along with the fact that their β-glucuronic acid substrates create hydrophilic chemistries, this is an attractive linker class. The substrate can be ligated to the antibody via a PABC spacer and is thus often combined with hydrophobic payloads. These ADCs could achieve DAR ratios of 8.3 without extreme aggregation and proved to be remarkably stable in rat plasma. Furthermore, high DARs with relatively low lipophilicity often show low clearance and good efficacy. Branched PEG linkers can be used to further enhance hydrophilicity. Unfortunately, various studies revealed relative low maximum tolerated doses for yet unknown reasons (S2).

Figure 6.5: Cleavage mechanisms of β-glucuronic acid- containing conjugates (S2).
6.1.2.3 Phosphatase cleavable linkers

Acid (pyro)phosphatase, also present in lysosomes, are also explored for ADC cleavage. These enzymes enable hydrolysis of (pyro)phosphates to release the parent alcohols. This method benefits from highly hydrophilic substrates and the possibility release of OH-containing payloads. A dual-enzyme labile linker was developed consisting of valine-citirulline-PAB chemistry with a (pyro)phosphate as leaving group. First, proteases attack the dipeptide releasing a (pyro)phosphate-drug, followed by phosphatase-mediated hydrolysis of the latter into an alcohol-drug (figure 6.6). However, this should be interpreted with caution as it is only assumed that these enzymes are responsible for the linker degradation and we are still waiting for in vivo results. Unfortunately, plasma stability was poor (52).

![Figure 6.6: Cleavage mechanism of a dipeptide-phosphate-conjugate (52).](image)

6.2 NON-CLEAVABLE LINKERS

Non-cleavable linkers release their payload during lysosomal proteolytic degradation after internalization and are less prone to non-specific release in plasma. Often there will remain charged amino acids on the payload changing cell permeability or causing a different cytotoxic effect. Thus, bystander effect (see section 8.2.2) is limited and the selection and structure of a payload must be careful and deliberate so that the released, modified payload form exhibits equal or greater activity (35). All possible metabolites should be further investigated to estimate their activity and toxicity. Successful examples of non-cleavable linkers include thioether and maleimide-based moieties as used in ado-trastuzumab emtansine and belantamab mafodotin (7). Two prevalent maleimide-linkers (figure 6.7), maleimidocaproyl (mc) and maleimidomethyl cyclohexane-1-carboxylate (mcc), also often used as spacers in addition to linkers sensitive to cleavage outside the target cell. Again, retro-michael deconjugation is best avoided by using a self-hydrolysing maleimide linker (39).

![Figure 6.7: Visualisation of two common maleimide-based linkers: maleimidocaproyl (mc)(A) and maleimidomethyl cyclohexane-1-carboxylate (mcc) (B) (39).](image)
7 PAYLOAD

Highly potent drugs are required for this application, with an IC$_{50}$ under $10^{-10}$ M. These drugs are a 100 to a 1000 times more cytotoxic than classical chemotherapeutics such as methotrexate and doxorubicin (3). Furthermore, the hydrophobicity of the payload is thought to play a crucial role in the bystander effect (see section 8.2.2) (7). A relative comparison of the payloads used for ADCs can be found in figure 7.1 (53).

![Figure 7.1: Relative comparison of payload potency.](image)

7.1 MICROTUBULE-TARGETING DRUGS

Microtubules consists of αβ-tubulin heterodimers (figure 7.2) forming highly dynamic protein polymers that are a part of the cytoskeleton in our cells. Several cellular processes depend directly or indirectly on the optimal functioning of the microtubules including mitosis, cell motion and organelle transport (54). Microtubuli can be seen as hollow tubes that starts growing from a centrosome when the α-end (minus end) of a αβ-tubulin dimer binds with a γ-tubulin that is part of the centrosome. The microtubule then grows further from the β-end (plus end). On each αβ-tubulin dimer a guanosine triphosphate (GTP) is bound and can be hydrolysed to guanosine diphosphate (GDP). As long as the plus end tubulin dimers carry GTP molecules (GTP cap) the growth process will continue. However sporadically and spontaneously a GTP in the GTP cap hydrolyses to a GDP leading to rapid microtubule depolymerization. Vice versa, a tubulin dimer can bind to a new microtubule by changing GDP to GTP. This process is known as the dynamic instability of microtubules, hence their highly dynamic nature (55).

![Figure 7.2: Formation of microtubule (56).](image)

Two classes of microtubule-targeting drugs belonging to the family of vinca domain binding molecules will be discussed. They attack microtubules by interfering with the lateral interaction between tubulin dimers, sabotaging their normal function. Because of the essential role microtubules play in cell division the cell is now forced to remain in metaphase which ultimately leads to apoptosis. At high concentrations, which are never
reached in clinical practise, these drugs could also depolymerise the microtubule by further reducing the interaction between tubulin dimers. A second mechanism by which vinca domain binding molecules disturb normal functioning of microtubules is by inhibition of nucleotide exchange and hydrolysis (54).

7.1.1 Auristatins

A first and most commonly used class of warheads are the Auristatins, known as synthetic microtubule destabilizers. Their chemical structure is based on dolastatin 10, a naturally occurring pentapeptide formed by a species of large sea slug called Dolabella auricularia (figure 7.3) (54,57). Monomethyl auristatin E and F (MMAE and MMAF) represent this class on the ADC market. Both still contain five peptides, but the N-terminus was demethylated to a secondary amine allowing linkage to an mAb. Also, the terminal dolaphine moiety was replaced by norephedrine (MMAE) or phenylalanine (MMAF) making the synthesis easier and less expensive but keeping the β-phenethylamide moiety which is considered essential for activity (55,58). The small structural difference between MMAE and MMAF (figure 7.3), a hydroxyl group versus a carboxylic acid group, causes for a difference in charge. MMAF is negatively loaded, and therefore it cannot pass through the cell membrane as a free drug, making free-MMAE more effective. However, this does not matter once MMAF is linked to an mAb, because the entire molecule can now only enter the cell through internalisation and when MMAF is released as a free drug it will be even more potent than MMAE. This is on the grounds that the carboxylic acid group causes for better interaction with tubulin. Note that free intracellular MMAF will be trapped so that it cannot cause toxicity by diffusion to healthy cells, probably making these conjugates less toxic. On the other hand, free MMAE might lead to the described bystander killing effect as the neutral agent can cross the membrane and kill neighbouring cells (55).

Figure 7.3: Structures of dolastatin 10, monomethyl auristatin E and F (MMAE) (MMAF) (59).
7.1.2 Maytansinoids

Maytansinoids, derivatives of maytansine found the bark of the African shrub called *Maytenus ovatus*, are considered a second class of microtubule-targeting drugs (60). Maytansine is reported to be a vinca domain binding drug and has been evaluated for clinical efficacy but was found to suffer from an unacceptably low therapeutic index. After these findings, interest in maytansine declined until ImmunoGen Inc. developed synthetic analogues (figure 7.4) which could be linked to an antibody and which have higher potency than the parent molecule. These analogues are currently known as drug maytansinoids (DMs) with DM1 [N-deacetyl-N-(3-mercapto-1-oxopropyl)-maytansine] used in trastuzumab emtansine (61).

![Figure 7.4: Structures of maytansine (parent molecule) and synthetic derivatives: DM1, DM2, DM3 (42).](image)

7.2 DNA-DAMAGING AGENTS

DNA-damaging agents are known for their higher potency and for their toxic effect in both dividing and non-dividing cells (51).

7.2.1 Calicheamicins

Calicheamicin (figure 7.5) was originally isolated from actinomycetes bacteria and based on this structure the semi-synthetic derivative ozogamicin was developed, N-acetyl-g calicheamicin 1,2-dimethyl hydrazine dichloride (62). After internalisation of the antibody-drug conjugate and release of ozogamicin, the warhead is then activated through reduction of the disulphide bond. This is triggered by a strong nucleophile, probably glutathione. Ozogamicin is an enediyne drug meaning it contains two triple bonds surrounding one double bond. After activation the enediyne presumably binds to the minor groove in DNA whereby the double-strand breaks leading to apoptosis (63).

![Figure 7.5: Illustration of a calicheamicin with its notable enediyne (53).](image)
7.2.2 Camptothecin analogues

Camptothecin was discovered in 1966 and first isolated from the stem bark of *Camptotheca acuminata*, a native tree from China (64). This natural product proved to inhibit topoisomerase I (TOPO 1), an enzyme involved in, among other things, DNA transcription and replication. TOPO 1 is able to cleave one of the two strands in double stranded DNA, unwind it partially and reanneal in order to relax the strand. Camptothecin and its analogues can interact with the TOPO 1/DNA complex, thereby preventing reannealing which ultimately leads to cell death because of partially cleaved DNA accumulation. Camptothecins have a minimum of five rings (A-E) and the lactone ring can open up in vivo as illustrated in figure 7.6, losing part of its activity. This can be explained by the pronounced (>99.9%) plasma protein binding and inferior cell diffusion because of its negative charge (65).

![Diagram of Camptothecin](image1)

*Figure 7.6: The cytotoxic lactone agent DXd (left) in equilibrium with its open form (right) (65).*

Dxd (figure 7.7), a derivative of exatecan, was the first camptothecin to be used in a commercial ADC. This compound has a fluorine at C11, believed to boost its cytotoxicity, and an additional F-ring thereby increasing lactone stability (65). SN-38 (figure 7.7) is another semi-synthetic camptothecin and a rather atypical warhead because of its significant lower potency than the other cytotoxic agents (figure 7.1) with an IC\(_{50}\) around 1.0-6.0 nM against multiple human cancer cell lines. SN-38 is the active metabolite of irinotecan, a classical chemotherapeutic and therefore has the important advantage of a well-known pharmacology (53). Furthermore, sacituzumab govitecan, the ADC which comprises SN-38, has a relatively high DAR without negative impact on the pharmacokinetics and antigen targeting of the conjugate (66). Although the pharmacology of the cytotoxic agent is known it is complicated by in vivo glucuronidation by UGT-1A to the inactive SN-38G and opening of the lactone function. The compound is also highly hydrophobic with minimal possible coupling sites, thereby making the construction of an ADC without lowering its activity challenging (53).

![Diagram of SN-38 metabolism](image2)

*Figure 7.7: Metabolization sites of prodrug irinotecan and its parent compound SN-38 (66).*
7.2.3 Pyrrolobenzodiazepines

Pyrrolobenzodiazepines (PBDs) are remarkably potent cytotoxic agents first discovered as anthramycin in *Streptomyces* during the 1960s (67). Their structure (figure 7.8) is characterised by a functionalized aromatic (ring A) and a diazepine (ring B) bound to a pyrrolidine (ring C). The chiral centre (S) at site C11a allows the molecule to fit into the minor groove of dsDNA, while the electrophilic N10-C11 allows for a possible attack by guanine. These molecules are known to selectively bind a particular DNA sequence through two sequential steps. First, they rapidly and reversibly bind in the minor groove of the double stranded DNA through hydrogen binding, van der Waals and electrostatic attractions. This step is repeated until a low-energy region is found featuring a specific sequence containing guanine. Secondly, if the nucleophilic C2-NH2 (guanine) attacks the C11 of the PBD a covalent bond is formed. This step is slower, depending on the DNA sequence and PBD structure, ranging from 3 to 24 hours. The resulting PBD-DNA adducts can now induce apoptosis through a number of mechanisms including DNA strand cleavage, counteraction of DNA processing enzymes, specific transcription factors and some signaling pathways. Because there is often an upregulation of these transcription factors and signalling pathways along with a defective repair mechanism against the PBD-DNA adducts in tumour cells, an additional selectivity is acquired (68). Since the discovery of the first PBD, several synthetic variants have been developed as well as PBD dimers, ligated through a C8/C8' linker (figure 7.8). Since these contain two alkylating groups, there is a possibility for inter- and intrastrand crosslinking next to monoadducts. Interstrand adducts are assumed to be the most effective. Furthermore, due to supplementary adducts and their better stabilization, PBD dimers exhibit improved cytotoxicity (67).

![Figure 7.8: SARs for a PBD (A) and a PBD dimer ligated through an 8'/8' linker (B) (68).](image-url)
7.3 INNOVATION

Standard cytotoxic payloads are being traded for more innovative options such as targeted or immunostimulatory agents. For example, tumours strive to be immortal and manipulate cell mechanisms such as the overexpression of BCL-XL, an anti-apoptotic protein. Therefore, an inhibitor can directly target apoptotic pathways. At this moment, an ADC linked to a BCL-XL inhibitor warhead, mirzotamab clezutoclax, is enrolled in early phase clinical trials (NCT03595059) (69). Chemokines, Toll-like receptor agonists or STING agonists are warheads used for recruitment and/or activation of immune effector cells to target certain antigens associated to the tumour. This reinforces the immunological effect of the antibody backbone (7,70,71). Furthermore, cytotoxic radioisotopes and oligonucleotides which are able to interfere during translation in vivo are being exploited as novel warheads (72,73).

In an attempt to improve ADC activity, well considered therapy combinations are currently undergoing early phase clinical trials. A first promising strategy is to boost ADC delivery using, for example, the monoclonal antibody bevacizumab which targets the vascular endothelial growth factor A (VEGFA). It is thought that such antiangiogenic therapies might boost ADC delivery or even increase ADC activity (74). Another strategy uses additional agents to influence antibody target expression and/or processing. By inducing target overexpression ADC activity might increase by promoting ADC interaction. For example, BRAF/MEK inhibitors induce receptor tyrosine kinase AXL transcription leading to elevated efficacy (75). On the other hand, by stimulating target turnover or degradation, ADC uptake and payload release is promoted. For example, irreversible kinase inhibitors directed against HER2 can benefit its internalization rates (76). Nonetheless, it is important to take into account that these agents might also have an impact on the antigen expression in non-tumour cells. A third method is already applied in three of the nine ADCs on the market, combining the ADC with a classical chemotherapeutic agent characterized by a different mechanism of action. By adding such a systemic drug with distinct action there is a possibility of bypassing tumour heterogeneity considering its effect on target antigen-negative cells (7). Another approach might be to combine an ADC with immunotherapy, in fact there are currently more than 20 clinical studies ongoing. It is hoped that this might enhance the immunity reaction that is already partly ADC-mediated even against tumours that previously were not prone to the immunity system (77). And finally, the combination with a drug that can counteract resistance mechanisms has also potential, for example, the use of polo-like kinase 1 (PLK1) inhibitors in T-DMI resistant tumours (78). Nevertheless, the combination of an ADC and an additional drug should always result from rational research based on more than sufficient preclinical data because all clinical studies pose a risk of additional or overlapping toxicities to the patients (7).
8 MECHANISM OF ACTION

Antibody-drug conjugates have a complex and versatile mechanism of action that to this day, is only partly understood. They combine the strengths of antibodies and ultrapotent cytotoxic agents causing unique pharmacodynamic and -kinetic properties. Unlike most first-line oncological therapies, ADCs must be processed by the tumour microenvironment before displaying their lethal effects and can thus be considered as prodrugs. The biochemical interaction of ADCs can be simplified to, in chronological order, binding of the antibody to the target antigen, internalisation and intracellular payload release. However, the actual situation is more complicated and varies significantly among ADCs (7).

8.1 PRIMARY MECHANISM OF ACTION

8.1.1 Antibody-antigen interaction

The ADC formulation administered to the patient contains three main components, i.e. the conjugate and in a lesser extent the naked antibody plus the free toxic payload. Dependent on linker stability and product purity, the composition of the formulation may differ between ADCs and the relative ratio of the three compounds may alter later on in vivo. Obviously, there should be as minimal as possible fraction of free payload (7). The latter has been observed in clinical pharmacokinetic studies with trastuzumab emtansine (T-DM1) (79). Here peak serum concentrations of the conjugate plus naked mAbs was 20\% higher than the conjugate alone, whereas the concentration of free payload was several orders of magnitude lower. Additionally, the half-life of the conjugate plus naked mAbs was around 9-11 days compared to 4 days for the conjugate alone. This could be explained by hepatic clearance of the complete conjugate or instability of the linker. However, these findings indicate the dynamic character of the component ratios in vivo and certainly underline the complication of pharmacological modelling and influence on clinical characteristics (7). Delivery of ADCs to tumours is complicated due to their size and deviant characteristics of the tumour environment, including vascularly anatomy, transcapillary pressure gradients and stromal tissue component. ADCs reach the tumour site dependant on the blood flow to the tissue. After the conjugates leave the capillaries (extravasation), they ultimately reach the tumour cells through passive diffusion leading to slow, inefficient and heterogeneous penetration of the tissue (figure 8.1, step 1 and 2) (7). Furthermore, a phenomenon called “binding-site barrier” has been described following the use of high-affinity antibodies targeting high-density and high-turnover targets. This phenomenon results in reduced penetration in the inner regions of the tumour tissue because of the rapid binding to the more accessible antigens. This issue is mostly seen when the bystander effect is absent and in this case distribution to perivascular regions can be promoted through co-administration of the naked antibody. Because competition at the binding site enhances homogenous distribution (80). Based on labelled mAb studies and mathematical modelling it is assumed that only a small percentage of the total ADC reaches their target. Likewise, based on
labelled mAb studies in animal models alone it is believed that it takes one to two days for ADCs to reach their maximum concentration at the tumour site, but it needs to be noted this may vary between ADCs (7).

8.1.2 Internalisation
A next step towards payload delivery for most conjugates is internalisation (figure 8.1, step 3) of the ADC-antigen complex. A first and popular way is antigen-dependent endocytosis. This is mainly clathrin-mediated, a well-documented process starting after association with the ligand for most ADCs (81). The ADC-antigen complex then recruits adaptor and clathrin proteins that form a clathrin-covered cavity. After maturation it is cleaved from the plasma membrane using membrane-binding and cleavage proteins, forming a clathrin-coated vesicle subsequently dismantled and usually fused with an early endosome (82). Unfortunately, there is also the possibility of antigen-independent uptake, through pinocytosis. This mainly occurs at excessive ADC concentrations, after saturation of target antigens, causing worse side effects (81).

8.1.2.1 FcRn salvage pathway
Endogenous immunoglobulins are characterised by long serum half-lives induced by the neonatal Fc receptor (FcRn), thus also affecting the antibody backbones of ADCs (figure 8.1, step 4). FcRn counteracts intracellular degradation after target antigen binding and internalization by transporting the endosome back to the cell-surface. The reduced pH of endosomes enables binding of two FcRn molecules to IgG after which the excessive unbound IgG is further transported to the late endosomes and/or lysosomes for degradation. The FcRn-IgG complex is disintegrates when it returns to the extracellular level at a higher pH (83). This depends on both the antibody and the antigen (81).

8.1.3 Payload release
Next, ADC-antigen complexes are trafficked through the endosomal and/or lysosomal pathway. These vesicles are functionalized with H⁺-ATPase proton pumps establishing a low pH. Based on studies inhibiting H⁺-ATPase or neutralizing these vesicles, it is believed that this trafficking is dependent on organelle acidification. If not recycled, the complex remains in the endosome until fusion with another endosome or with trans-Golgi-network vesicles. These contain proteases and hydrolases that can optimally degrade the ADC-antigen complex at low pH (figure 8.1, step 5). Now a late endosome has formed that may further fuse with a lysosome. Because the ADC-antigen complex is too voluminous and hydrophilic, it is only after release of the payload that diffusion through the vesicle is possible on route to its target (figure 8.1, step 6 and 7). If payloads are released in early endosomes or late endosomes/lysosomes depends on the type of linker. This whole process, from ADC-antigen interaction all the way to payload release can take more than 24 hours (7,81).
8.2 ADDITIONAL MECHANISMS OF ACTION

8.2.1 Extracellular payload release
Extracellular payload release (figure 8.1, step 2A) can occur with most linker types when exposed to the tumour microenvironment that already has a low pH, contains proteases or a redox potential. However, this can be beneficial for the activity on target antigen-negative malignant cells that are otherwise left untargeted and therefore strategies have been developed to intentionally and selectively release the payload in the TME. First, utilization of a cleavable linker plus a cell membrane permeable drug is the obvious choice to enable extracellular release. Indeed, it was demonstrated that cleavable linkers were still effective when targeting non-internalizing antigens in contrast with non-cleavable linkers. Second, non- or poor-internalizing target antigens, previously unconsidered, can be selected with higher selectivity and expression levels. And third, to further combat tumour heterogeneity, target antigens found in the TME or tumour vasculature are investigated. This would especially be beneficial for heterogenous and/or stroma rich solid tumours where the cell-surface of the tumour cell is hard to reach (84).

8.2.2 Bystander effect
The so-called “bystander effect” occurs when a free, lipophilic cytotoxic agent is able to cross the cell membrane and thus able to attack neighbouring cells, again, regardless of antigen expression and after tumour penetration (figure 8.1, step 6A). Especially tumours with heterogeneous antigen expression benefit from this phenomenon. Cleavable linkers are preferred above non-cleavable to generate an uncharged payload and to promote this bystander effect. The relative level and speed of the bystander effect are elevated by higher proportions of antigen-positive cells, resulting in less and less bystander effect over time as the cytotoxic agent acts upon the antigen-positive cells. This was examined in preclinical studies by alternating the relative proportions in co-culture and suggests that antigen-positive cells are necessary for ADC processing and payload release. Of note, it is believed that the more payload gets released intracellularly, the less the bystander effect depends on these factors. However, it is probable that this effect also brings along increased toxicity when it acts upon non-malignant cells or immune mediators. This is not well documented and needs to be further examined (7).

8.2.3 Payload-independent anti-tumour activity
Because payloads are linked to the antibody at locations outside of the antigen-recognition site antigen affinity will typically be maintained. Therefore, the ADC can exert an immunological effect on the malignant cell through its antibody part often before payload release. This immunological effect is mediated through the antigen binding fragment (Fab) and the crystallizable fragment (Fc) of the antibody. Whereas Fab-activity can lead to inhibition of ligand binding and dimerization, inducing endocytosis and antigen degradation, the Fc domain can cause antibody-dependant cellular cytotoxicity (ADCC), complement-dependant cytotoxicity (CDC) and/or
antibody-dependant cellular phagocytosis. Antibodies targeting a highly expressed oncogenic antigens are preferred above those targeting antigens that play no significant role in the oncogenic pathway. These payload-independent MOAs are noted in figure 8.1, step 2B (7).

2B. Payload independent anti-tumour activity
Fc-mediated stimulation of immune cell effector functions. Disruption of receptor dimerization, function or downstream signaling.
After tumour cell degradation the free payload will be released in the TME. If cell membrane permeable, it can exert its effect on the surrounding cells.

2A. Extracellular payload release
Before internalization, linker cleavage can already occur due to prevailing TME conditions leading to payload release. If cell membrane permeable, it can exert its effect on the surrounding cells.

2. Binding
mAb component of the ADC binds to the target antigen in the cell surface.

3. Internalization
ADC-antigen complex is internalized mainly target-dependent through mainly clathrin-mediated endocytosis.

4. Recycling
A fraction of ADCs in the early endosome bind to FcRn receptors and get transported back out of the cell.

5. ADC degradation
Endosomal/lysosomal trafficking leads to degradation through acidic or enzymatic cleavage.

6. Drug release
The free payload can now diffuse out of the vesicle.

6a. Bystander effect
Cell membrane permeable drugs can diffuse out of the cell to "bystander" cells regardless of target antigen expression and exert their toxic effect.

7. Action
The cytotoxic agent interferes with critical cellular machinery such as DNA or microtubule assembly, resulting in cell apoptosis.

Figure 8.1: Illustration of the mechanism of action of an ADC with further explanation of the classical MOA in black and additional MOAs in grey (85).

8.3 Clinical efficacy
After decades of research and tremendous effort to improve ADC constructs, the question remains whether antibody-drug conjugates offer a benefit in cancer treatment or not. What is the relation between ADC molecular
structure and macro-level activity and toxicity observed in patients (7)? The objective response rate (ORR) is a frequently applied method for measuring treatment effect in tumours during clinical trials. The FDA definition of ORR is described as “proportion of patients with a tumour size reduction of a predefined amount and for a minimum period of time” (86). In the following, we will touch on a selection of clinical studies to get an idea of the ultimate ADC effect demonstrated by this ORR.

Trastuzumab emtansine (T-DM1) achieved an ORR of 43.6% in a randomised trial for patients resistant to the therapeutic mAb trastuzumab and taxane, which like DM1 is also a microtubule-targeting chemotherapeutic. This was examined in HER2-positive advanced breast cancer. The ADC was favourably compared with the dual small-molecule therapy with lapatinib and capecitabine which showed an ORR of 30.8% (87). Furthermore, trastuzumab deruxtecan (T-DXd), a next-generation ADC also targeting HER2, gave an ORR of 60.9% in patients with metastatic HER2-positive breast cancer that were not cured with previous treatment of T-DM1. These results were conducted in a non-randomised, phase 2 trial. This might be explained by the different advantageous characteristics of T-DXd such as a cleavable linker (bystander effect), relative high DAR (8 compared to 3.5) and a TOPO-1 inhibitor as payload. This DNA-damaging mechanism of action is barely used in early-stage breast cancer compared to microtubule inhibitors. Thus, the payload of T-DM1, a microtubule inhibitor, will probably be more prone to resistance (88). Furthermore, T-DXd exhibited an ORR of 51% in a phase 2, randomised trial studying gastric cancer compared to 14% for chemotherapy with another TOPO-1 inhibitor, irinotecan (89).

Sacituzumab govetican, a TROP2 targeting ADC with a TOPO-1 inhibitor as payload was examined in a randomised phase 3 trial for refractory metastatic triple-negative breast cancer compared to a single-agent therapeutic of a physician’s judgement (eribulin, vinorelbine, capecitabine, or gemcitabine). The ADC showed an ORR of 35% compared to 5% for chemotherapy. Additionally, progression-free and overall survival were significantly longer (90). Enfortumab vedotin was also investigated in a single-arm phase 2 trial with metastatic urothelial carcinoma as indication. All enrolled patients were previously treated with platinum chemotherapy and checkpoint inhibitor drugs. The study confirmed an ORR of 44% for the ADC (91).

These findings reinforce the suspicion that no single aspect of ADCs is solely responsible for their observed activity, instead the simultaneous alterations in drug potency, mechanism of action and tumour delivery probably all play a role. Moreover, poor efficacy of a certain cytotoxic drug does not prevent ADC payloads with a comparable target or MOA from generating a satisfactory response. Because enhanced antibody-dependent drug delivery to the tumour might benefit activity of these cytotoxic drugs. In conclusion, it is anticipated that the efficacy of an ADC is determined by both antibody, linker and payload dependent factors, all of those being a function of complicated interactions among the ADC and numerous components of the tumour as well as the tumour microenvironment (TME) (7).
9 TOXICITY

9.1 TOXICITY PROFILES

The toxicity profile of an ADC is tough to predict based on its composition only because of the complex factors described below. Therefore, therapeutic drug monitoring (TDM), close dose selection and attentive AE reporting are recommended while additional explanatory preclinical and translational studies are obviously needed. These toxic effects on healthy cells (off-tumour) can be divided into two groups, depending on whether there is interaction with the target antigen or not (off/on-target) (7).

9.1.1 On-target, off-tumour toxicity

ADCs are designed to deliver their payload selectively to cells after interacting with their “unique” target protein. However, these proteins often also occur on normal cells to a lesser extent. Nevertheless, this may contribute to the toxicity because the expression pattern of the antigen determines the distribution and accumulation of the toxic drug. As an example, three ADCs targeting different antigens, but which are otherwise equal, do not have the same toxicities. Enfortumab vedotin, used for urothelial carcinoma, binds to nectin 4, a target antigen also found in the salivary glands. This conjugate caused dysgeusia in 40% of the patients, a toxicity that has not been observed for brentuximab vedotin or polatuzumab vedotin (7).

9.1.2 Off-target, off-tumour toxicity

Off-target, off-tumour toxicities (table 9.1) contribute most to the overall toxicity of the current ADCs and are thought to mainly result from unintended payload release in the circulation, tumour microenvironment and non-malignant tissues. Another explanation could be ADC-uptake irrespective to the target antigen through binding to Fc receptors, macro- and micro-pinocytosis, which inspired researchers to increase the therapeutic index by reducing Fc receptor affinity. It is striking that some adverse effects occur independent from the mechanism of action of the payload, for instance MMAF induces ocular toxicities and MMAE does not, while they both belong to the auristatins class. These within-class differences show that minor chemical changes in linker or payload structure can have a major impact. Because MMAF is negatively charged in contrast to lipophilic MMAE it was reasoned that MMAF accumulates in corneal epithelial cells. However, both DM1 and DM4 have also often led to ocular toxicity independent from linker type. This is despite the fact that non-cleavable linkers provide a loaded payload upon release and cleavable linkers do not. So, it seems that these adverse effects are not unique to loaded drugs. This has led to a third and yet to be unravelled mechanism for off-target, off-tumour toxicities, whereby a single ADC causes different adverse effects dependent on tumour type. Glembatumumab vedotin caused severe rash for 30% of the melanoma-cases compared to 4% in patients with breast cancer, while the same dose regimen was used. Experts suggested that this might be provoked by priming of the immune system against proteins related to the tumour (7).
Table 9.1: Overview of off-target, off-tumour toxicities (independent of target antigen) based on meta-analysis of available data.

<table>
<thead>
<tr>
<th>Payload</th>
<th>Off-target, off-tumour toxicities</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMAE</td>
<td>Anaemia</td>
</tr>
<tr>
<td></td>
<td>Neutropenia</td>
</tr>
<tr>
<td></td>
<td>Peripheral neuropathy</td>
</tr>
<tr>
<td>MMAF</td>
<td>Ocular toxicity</td>
</tr>
<tr>
<td>DM1</td>
<td>Thrombocytopenia</td>
</tr>
<tr>
<td></td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>DM4</td>
<td>Ocular toxicity</td>
</tr>
</tbody>
</table>

9.1.3 Immunogenicity

In theory, ADCs carry a higher risk for immune reactions compared to therapeutic mAbs given the prevalence of non-natural elements. Therefore, 8 different valine-citrulline-MMAE ADCs indicated for various indications were studied across 11 clinical trials. Incidence of antidrug antibodies (ADAs) were comparable as for mAbs and were mostly directed against the antibody backbone of the ADC. Based on the acquired data, it was concluded that the ADC hapten-like structure did not seem to elevate immune responses in patients against these specific valine-citrulline-MMAE ADCs compared to mAb therapies (92). Moreover, the incidence of ADAs after gemtuzumab ozogamicin was less than 1% in four clinical studies (93). Of note, immunogenicity risk assessments should be conducted at an early stage of drug development and updated based on initial clinical data (92).

9.2 RESISTANCE

The mechanisms of resistance to ADCs are barely uncovered, but primary evidence indicates that the tumour cell can counteract the effects of an ADC via several mechanisms, including alterations in antibody-antigen interaction, conjugate internalization and processing or payload activity. Further investigation is necessary as it can help to better understand the mechanism of drug action and to identify predictive biomarkers (7). However, it is stated that resistance mechanisms of ADCs are more similar to those of chemotherapeutics than to mAb therapies. This is supported by the fact that mutations in PIK3CA confer resistance to trastuzumab but not to T-DM1 (7).

9.2.1 Primary or de novo resistance

Primary resistance, where therapy is ineffective from the start, can be countered by patient selection based on target expression levels. Because tumour heterogeneity is a major cause of resistance, such personalized treatment is beneficial especially for treatment of solid tumours, which often have a dynamic and a highly diversified target antigen expression. However, only two out of the four ADCs approved for treatment against
solid tumours, T-DM1 and T-DXd, are administered exclusively to patients who possess the target antigen in their tumour tissues. These conjugates both target HER2, a protein with an expression level that can differ by <5 logs in breast cancer because of high-level ERBB2 gene amplification. This HER2-positivity is defined by the guidelines of the ASCO-College of American Pathology through immunohistochemistry (IHC) plus fluorescent in situ hybridization. Of note, this strategy was designed for selecting patients for the monoclonal antibody trastuzumab and the added advantage of this strategy for ADCs specifically has not been documented. In fact, T-DM1 has proven efficacy in lung cancers with no overexpressed HER2 or with mutated HER2 target antigens, and T-DXd together with the experimental ADC trastuzumab duocarmazine have shown early signs of efficacy when administered to breast cancer patients with an expression level under the threshold for HER2 positivity. In favour of patient selection, glembatumab vedotin proved to be significantly more effective (ORR of almost 30% versus 6%) during a clinical breast cancer study when the expression level of gNMB, the target antigen, was ≥ 25% instead of ≥ 5%. This was measured by IHC. So, there is an urgent need for adequate predictive biomarkers and currently IHC is the primary method to measure expression levels of target antigens. Unfortunately, IHC is only a semi-quantitative assay and the thresholds are often intuitively defined without a clear rationale. However, this is a difficult task considering that the minimal occurrence of a cell-surface antigen needed for ADC activity can vary enormously and afterwards ADC activity might not correlate with the level of antigen expression. But most of all, tumour sensitivity can’t be measured through expression levels of target antigens alone as target turnover, heterogeneity, oncogenicity, TME properties, off-tumour occurrence of antigens… all affect the toxicity and efficacy of ADCs (7).

9.2.2 Secondary or acquired resistance

ADCs are believed to acquire resistance through multiple complex mechanisms based on numerous in vitro studies. Nevertheless, this has yet to be proven in human as only a minimum of clinical studies has been conducted to support these findings. A first possibility is for the cell to downregulate its target protein, thereby decreasing ADC interaction and intracellular payload release. The cell can degrade some of the cell-surface antigens in the lysosome and inhibit the gene form encoding (figure 9.1) (94).

Figure 9.1: Antigen downregulation and loss (7).
Furthermore, linker cleavage can be inhibited through modification of the lysosomal environment (modified lysosomal acidification, redox environment, proteolytic processes...) and elevated endosome recycling can result in extracellular release of the conjugate prior to payload release (figure 9.2) (94).

**Figure 9.2: Modification of intracellular trafficking pathways and lysosomal conjugate processing (7).**

Additionally, tumour cells can upregulate their ATP-binding cassette (ABC) transporters thereby promoting active efflux of the cytotoxic agent (figure 9.3). ABC-transporters are no new obstacle as they are well-known for the efflux of classical chemotherapeutics. That said, not all ADC payloads are substrates to these efflux pumps but MMAE, DM1 and ozogamicin are particularly prone to this type of resistance (94).

**Figure 9.3: Payload resistance especially through efflux by ATP-binding (ABC) cassette transporters (7).**
OVERVIEW OF APPROVED ANTIBODY-DRUG CONJUGATES

10.1 GEMTUZUMAB OZOGAMICIN

<table>
<thead>
<tr>
<th>Target antigen</th>
<th>CD33</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb isotype</td>
<td>IgG4</td>
</tr>
<tr>
<td>Linker type (conjugation)</td>
<td>Cleavable (lysine)</td>
</tr>
<tr>
<td>Payload (class), payload action</td>
<td>Ozogamicin (calicheamicin), DNA cleavage</td>
</tr>
<tr>
<td>DAR</td>
<td>2-3</td>
</tr>
<tr>
<td>indication</td>
<td>CD33+ R/R AML(^a) (2000) (2017)</td>
</tr>
</tbody>
</table>

\(^a\) acute myeloid leukaemia

10.2 BRENTUXIMAB VEDOTIN

<table>
<thead>
<tr>
<th>Target antigen</th>
<th>CD30</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb isotype</td>
<td>IgG1</td>
</tr>
<tr>
<td>Linker type (conjugation)</td>
<td>Cleavable (interchain cysteine)</td>
</tr>
<tr>
<td>Payload (class), payload action</td>
<td>MMAE (auristatin), Microtubule inhibitor</td>
</tr>
<tr>
<td>DAR</td>
<td>4</td>
</tr>
<tr>
<td>indication</td>
<td>R/R sALCL(^b) or cHL(^c) (2011), R/R pcALCL(^d) or CD30+ MF(^e) (2017), cHL, sALCL or CD30+ PTCL(^f) (2018)</td>
</tr>
</tbody>
</table>

\(^b\) systemic anaplastic large cell lymphoma;  
\(^c\) classical Hodgkin lymphoma;  
\(^d\) primary cutaneous anaplastic large cell lymphoma;  
\(^e\) mycosis fungoides;  
\(^f\) peripheral T cell lymphoma

10.3 ADO-TRASTUZUMAB EMTANSINE (T-DM1)

<table>
<thead>
<tr>
<th>Target antigen</th>
<th>HER2</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb isotype</td>
<td>IgG1</td>
</tr>
<tr>
<td>Linker type</td>
<td>Non-cleavable (lysine)</td>
</tr>
<tr>
<td>Payload (class), payload action</td>
<td>DM1 (maytansinoid), Microtubule inhibitor</td>
</tr>
<tr>
<td>DAR</td>
<td>3,5 (mean)</td>
</tr>
<tr>
<td>indication</td>
<td>Advanced-stage HER2+ breast cancer previously treated with trastuzumab and a taxane (2013), early stage HER2+ breast cancer in patients with residual disease after neoadjuvant trastuzumab-taxane-based treatment (2019)</td>
</tr>
</tbody>
</table>
### 10.4 OTHER APPROVED ANTIBODY-DRUG CONJUGATES

<table>
<thead>
<tr>
<th>ADC</th>
<th>Target antigen</th>
<th>mAb isotype</th>
<th>Linker type (conjugation)</th>
<th>Payload (class), payload action</th>
<th>DAR</th>
<th>Indication (year of approval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inotuzumab ozogamicin</td>
<td>CD22</td>
<td>IgG4</td>
<td>Cleavable (lysine)</td>
<td>Ozogamicin (calicheamicin), DNA cleavage</td>
<td>5-7</td>
<td>R/R B-ALL(^9) (2017)</td>
</tr>
<tr>
<td>Fam-trastuzumab deruxtecan-nxki (T-DXd)</td>
<td>HER2</td>
<td>IgG1</td>
<td>Cleavable (interchain cysteine)</td>
<td>DX(_d) (camptothecin), TOP01 inhibitor</td>
<td>8</td>
<td>Advanced-stage HER2+ breast cancer after two or more anti-HER2-based regimens (2019)</td>
</tr>
<tr>
<td>Polatuzumab vedotin-piiq</td>
<td>CD79b</td>
<td>IgG1</td>
<td>Cleavable (interchain cysteine)</td>
<td>MMAE (auristatin), Microtubule inhibitor</td>
<td>3.5 (mean)</td>
<td>R/R DLBCL(^b) (2019)</td>
</tr>
<tr>
<td>Sacituzumab govitecan-hziy</td>
<td>TROP2(^l)</td>
<td>IgG1</td>
<td>Cleavable (interchain cysteine)</td>
<td>SN-38 (camptothecin), TOP01 inhibitor</td>
<td>8</td>
<td>Advanced-stage, triple-negative breast cancer in the third-line setting or beyond (2020)</td>
</tr>
<tr>
<td>Enfortumab vedotin-ejfv</td>
<td>Nectin 4</td>
<td>IgG1</td>
<td>Cleavable (interchain cysteine)</td>
<td>MMAE (auristatin), Microtubule inhibitor</td>
<td>4</td>
<td>Advanced-stage urothelial carcinoma, following progression on a PD-1 or PD-L1 inhibitor and platinum-containing chemotherapy (2020)</td>
</tr>
<tr>
<td>Belantamab mafodotin-bilmf</td>
<td>BCMA(^j)</td>
<td>IgG1</td>
<td>Non-cleavable (cysteine)</td>
<td>MMAF (auristatin), Microtubule inhibitor</td>
<td>Not known</td>
<td>R/R multiple myeloma in the fifth-line setting or beyond (2020)</td>
</tr>
</tbody>
</table>

\(^9\) B cell acute lymphoblastic leukaemia; \(^b\) diffuse large B cell lymphoma; \(^l\) tumour-associated calcium signal transducer 2; \(^j\) B cell maturation antigen

This overview was composed on the basis of various references: (2,7,33).
11 DISCUSSION

In this thesis, a complete overview of the ADC construct is given together with various innovative technologies for further improvement. Based on years of investigation, the combination of a humanized IgG1 backbone, a tumour-associated target, a cleavable linker and a highly potent payload directed against microtubules or DNA is typically favoured. This becomes apparent when looking at the approved ADCs and those currently in clinical trials (33). However, at the end of the chapters (antibody backbone, target selection, conjugation, linker and payload) it was often concluded that there is no one general optimal choice but each component of the ADC is best chosen in consideration of the total ADC construct and therapeutic context such as tumour type. One size does not fit all. Moreover, there is an almost infinite number of combinations possible for ADC design and yet similar target antigen or drug linker moieties are frequently encountered. Gemtuzumab ozogamicin and inotuzumab ozogamicin, both patented by Pfizer, even bear the exact same drug linker structure, both conjugated through lysine coupling. This is not surprising since each element of an antibody-drug conjugate is selected through a small-scale drug screen using in vitro or in xenograft models. This often implies that one antibody is tested with several selected linker-payload combinations (95). Thus, we should strive to multi-parameter optimization to get closer to the optimal combination to improve clinical activity by, for example, better antibody-antigen interaction. This is because monoclonal antibodies can differ in their antigen-binding abilities, in their capacity to trigger receptor dimerization, and in their effects on target internalization and in their Fc-activity even if they target the same antigen (7).

It is known that the toxicity profiles of ADCs are dominated by off-target, off-tumour toxicities due to unwanted payload release. This may be the result of plasma unstable linkers or antigen-independent internalization. For example, with older maleimide linkers, deconjugation often resulted in systemic toxicities. Thus, further optimization is needed to combat these dose-limiting side effects (96).

Although ADCs are primarily designed for, they are not limited to cancer therapy. The most successful example is an anti-*Staphylococcus aureus* Thiomab IgG1 antibody combined with an innovative antibiotic from the rifamycin class through a protease cleavable linker. Because bacteria (and viruses) do not possess mechanisms to directly internalize an ADC, activity depends ADC-*S. aureus* absorption by phagocytic cells, whereafter intracellular cathepsin B can degrade the linker. This is important since this bacterium can lead to infections known for their high morbidity and mortality while conventional antibiotics does not seem to adequately eradicate intracellular *S. aureus*. This ADC, named DSTA4637S, has reached phase 1 clinical trials. A randomised study proved that the ADC is generally safe and has a positive pharmacokinetic (PK) profile in healthy volunteers, encouraging further investigation (97). In addition, ADCs have also been designed against CXCR4, a protein found on T cells and in a lesser extent on other hematopoietic cells as treatment for chronic myelogenous leukaemia.
and certain forms of acute lymphoblastic leukaemia. This was linked to dasatinib which acts on tyrosine kinases but causes many side effects when administered orally. Promising in vitro studies have been reported but further research in humans is still needed, especially since the target antigen shows different levels and distribution in mice. Furthermore, because CXCR4 predominantly occurs on hematopoietic versus normal cells, the combination with tacrolimus is also investigated. This medicine is used for organ transplants or in certain autoimmune diseases to weaken the immune system. However, a very potent drug often comes with severe side effects, especially when used on a long-term basis. Successful in vitro studies demonstrated payload delivery into the target cells, resulting in significantly reduced pro-inflammatory cytokines (98).

We must not forget the economic side of the story either, what would be the market size of ADCs? According to Nature reviews, the global sales of currently approved ADCs will reach a striking US$16.4 billion by 2026. On the basis of this analysis, trastuzumab deruxtecan alone will contribute 6.2 billion. This is explained by its broad application, indicated for several subtypes of breast cancer and its relatively long treatment (99). However, the global pandemic caused by COVID-19 led to a market decline in the first half of 2020 due to supply chain interruption (100).

However, the ultimate question must remain: do ADCs offer any advantages over current and emerging cancer therapies and at what cost? To answer this question, more comparative studies will need to be conducted by measuring tumour response, quality of life and survival. As discussed in section 8.3, several studies showed a superior ORR, mostly towards chemotherapeutics.
CONCLUSION

The original idea behind ADCs, that antibody-mediated delivery of a cytotoxic agent will diminish its side effects and subsequently enhance its antitumour activity, continuous to be the guiding principle. While early failures can be explained by suboptimal technologies and a misperception of ADC complexity, upcoming ADC technologies, together with cumulative experience, offer innovative possibilities. This makes ADCs a more exciting therapeutic drug class than ever before.

Antibody backbone development has benefitted from overall improvement on therapeutic mAb design, thereby reducing unwanted immune responses against this exogenous molecule. A IgG1 backbone is most commonly used because of its superior payload-independent antitumour activity and its relatively easy production. To improve internalisation and/or tumour selectivity, bispecific ADCs are being explored. Furthermore, smaller targeting ligands are also entering clinical trials with the hope of enhancing tumour penetration leading to a more homogeneous drug distribution and complete tumour killing. A target antigen is typically selected based on overexpression and tumour selectivity. The following should also be considered: target turnover, oncogenicity and heterogeneity. Moreover, targeting non-internalizing antigens leading to extracellular payload release can combat heterogeneous solid tumours while targeting mutant proteins of the tumour can dramatically increase selectivity. Homogeneous bioconjugation methods are expected to exceed conventional lysine or cysteine conjugation due to enhanced manufacturing control and a more stable PKPD profile, considering that naked or overloaded antibodies can impact the PKPD profile. The choice of a stable, non-cleavable linker in contrast to a cleavable one, depends partly on target expression and on the tumour specificity of the target antigen. A cleavable linker can partly overcome low and/or heterogeneous target expression through the bystander effect. However, the potential toxic effect on healthy or immune cells should also be taken into account. Cytotoxic agents for ADC use can be categorised into microtubule or DNA damaging agents. These are extremely potent drugs because only a fraction of the administered dose makes it to the indentent tumour site. Fortunately, other cytotoxic agents are currently being investigated that can kill off the tumour cell through completely different mechanisms. Conjugation of two distinct payloads or combination therapy may also enhance the clinical effect. Furthermore, for a better patient selection, adequate tumour sensitivity measurements to ADCs must be developed since quantitively measurements of target expression does not capture the whole picture. Additionally, a better understanding of ADC action on a cell-specific and tumour-specific basis, including the role of the TME and resistance mechanisms might lay the foundation for a broader use of ADCs and a possible transformation of oncology treatment.
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