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A review on antibody drug conjugate: A new approach to cancer therapy

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Abstract
Antibody drug conjugates (ADCs) have emerged as an important class of anticancer therapeutics. They are designed to deliver highly cytotoxic small molecules directly to cancer cells via tumor specific antibodies, therefore providing a much wider therapeutic window than conventional chemotherapy. Although chemotherapy has seen great success in treatment of cancer, but severe adverse effects derived from off-target cytotoxicity may worsen a patient's quality of life, leads to discontinuation of medication. Antibody drug conjugates (ADCs) utilize a monoclonal antibody to deliver a cytotoxic payload specifically to tumor cells, limiting exposure to healthy tissues. It consists of a cytotoxic small molecule covalently linked to a targeted protein carrier (monoclonal antibody) via a stable cleavable or non-cleavable linker. Incomplete understanding of ADCs mechanism of action, its off-target toxicities, and difficulties in the selection of suitable clinical settings such as patient selection, dosing regimen are some possible explanations for the slow clinical translation of new ADCs. However, we expect to see a generation of safer and more effective ADCs for clinical translation and commercialization in the future (Shah, 2017).

Keywords: antibody drug conjugate, cancer therapy, chemotherapy

Introduction
Antibody drug conjugates (ADCs) have emerged as an important class of anticancer therapeutics (Hedrich *et al*., 2018) [1]. They are designed to deliver highly cytotoxic small molecules directly to cancer cells via tumor specific antibodies, therefore providing a much wider therapeutic window than conventional chemotherapy (Mecklenburg, 2018) [2]. In simple terms antibody-drug conjugates deliver 'deactivated' cytotoxins to specific cancer cells. Over the past half century, cancer management has improved significantly along with the advancement of chemotherapy (DeVita and Chu, 2008) [3]. Chemotherapy using cytotoxic agents is a major treatment option, in addition to surgical removal, radiation, targeted therapies using small molecules or monoclonal antibodies (An, 2010) [4] and more recently, immunotherapy. Although chemotherapy has seen great success in treatment of cancer, especially leukemia, but chance of development of resistance mechanisms is higher. Severe adverse effects derived from off-target cytotoxicity may worsen a patient's quality of life, leads to discontinuation of medication. So concept, more highly potent cytotoxic agents for cancer treatment was given by clinicians and medicinal chemist. (Tsuchikama and An, 2016) [5].

Antibody drug conjugates (ADCs) are biopharmaceutical molecules consisting ofa cytotoxic small molecule covalently linked to a targeted protein carrier via a stable cleavable or non-cleavable linker. The process of conjugation yields a highly complex molecule with biochemical properties that are distinct from those of the unconjugated components (Hinrichs and Dixit, 2015) [1]. Antibody drug conjugates (ADCs) utilize a monoclonal antibody to deliver a cytotoxic payload specifically to tumor cells, limiting exposure to healthy tissues (Masters *et al*., 2017) [6]. The hybrid nature of ADCs highlights the need for a science-based approach for safety assessment that incorporates relevant aspects of small and large molecule testing paradigms (Hinrichs and Dixit, 2015) [1].

The antibody drug conjugate field has made significant progress recently owing to careful optimization of several parameters, including mAb specificity, drug potency, linker technology and the stoichiometry pacement of conjugated drugs. The underlying for this has been obtained in pre-clinical bio distribution and pharmacokinetics studies showing that targeted delivery leads to high intratumoral free drug concentration, while non target tissues are largely

spared from chemotherapeutic exposure (Masters *et al*., 2017) [6]. To date total eight ADCs are approved by U.S. Food and Drug Administration (FDA): brentuximab vedotin (Adcetris®), gemtuzumab ozogamicin (Mylotarg®), ado-trastuzumab emtansine (Kadcyla®), inotuzumab ozogamicin (Besponsa®) and polatuzumab vedotin-piiq (Polivy®), enfortumab vedotin (Padcev®), trastuzumab deruxtecan (Enhertu®) and sacituzumab govitecan (Trodelvy®).

History

The origin of the antibody–drug conjugate (ADC) concept of has been attributed mostly to the "magic bullet" idea proposed by Paul Ehrlich more than 100 years ago. He demonstrated the selective absorption of dyes with different chemical structures by various tissues and thus targeted delivery of drug to the disease site is achieved. More importantly, Ehrlich was one of the scientists responsible for the discovery of antibodies and was the first one to describe the unique "receptors" on the target cells that could be recognized by antibodies (Strebhardt and Ullrich, 2008) [7].

Most serious challenge at that time was how to translate the studies with animal immunoglobulins into clinical applications. With an alkylating chemotherapeutic agent, it was demonstrated that covalent conjugation between the immunoglobulin and the drug is essential to achieve the tumor targeting effect (Ghose and Nigam, 1972) [8]. Daunomycin and Adriamycin could be linked covalently to anti-bovine serum albumin (BSA) immunoglobulins with various covalent reactions, but the retention of both drug and antibody activities was observed only with the periodate oxidation method (Hurwitz *et al*., 1975) [9]. This finding opened a new area in ADC, i.e., the linker chemistry, which played an important role in the later design of ADC (Blair and Ghose, 1983) [10]. However, Mylotarg® withdrawn from market because in 2010 due to lack of clinical benefit and increase fatal toxicity rate. Even though gemtuzumab ozogamicin was withdrawn from the market in 2010; it was a milestone in the clinical application of ADCas a therapeutic drug (Shen, 2015) [11].

Table 1: Paradigm of antibody drug conjugate development for cancer therapy

1913	Concept of selective delivery of toxic agents to target cell causing disease was originally proposed by German physician and Scientist Paul Ehrlich
1958	Targeted therapy was first demonstrated by Mathe in the form of an ADC, Methotrexate conjugated to a leukemia cell targeting antibody
1983	Ist ADC Human clinical trial was conducted using an anti carcino embryonic antigen antibody- vindesin conjugate
1990	First generation ADCs consisting of chimeric or humanized antibodies
2000	FDA approved Gemtuzumab ozogamicin (Mylotarg®) for CD33 positive acute myelogenous leukemia
2011	FDA approved Brentuximab vedotin (Adcetris®) for CD30 positive relapsed or refractory Hodgkin's lymphoma and systemic anaplastic large cell lymphoma
2013	FDA approved Trastuzumab emtansine (Kadcyla®) for breast cancer

Antibody Drug Conjugate (ADCS)

Antibody drug conjugates or ADCs are empowered antibodies (mAbs) designed to harness the targeting ability of monoclonal antibodies by linking them to cell-killing agents. An ideal ADC has:

- A highly selective monoclonal antibody (mAb) for a tumor-associated antigen that has restricted or no expression on normal (healthy) cells.
- A potent cytotoxic agent (generally a small molecule drug with a high systemic toxicity) designed to induce target cell death after being internalized in the tumor cell and released.
- A linker that is stable in circulation but releases the cytotoxic agent in target cells.

Mechanism of Action of ADCS

When designing an ideal ADC, it is essential to understand the mechanism of action in order to identify the desired features of each of its three components. Numerous pre-clinical efficacy studies have shown that ADCs enhance the antitumor activity of "naked" antibodies and reduce the systemic toxicity of the cytotoxic drugs conjugated to the antibody (Lambert, 2005) [12]. Each of the steps involved in the mechanism of action is associated with unique challenges (Ducry and Stump, 2010) [13] that complicate the design of ADCs.

ADCs are administered intravenously in order to prevent the mAb from being destroyed by gastric acids and proteolytic enzymes. The mAb component of the ADC enables it to circulate in the bloodstream until it finds and binds to tumour-specific (or tumour-associated) cell surface antigens present on target cancer cells (Nolting, 2013) [14]. Once the mAb component of the ADC is bound to its target antigen, the ADC– antigen complex internalized via receptor-mediated endocytosis. The internalization process finishes with the formation of a clathrin-coated early endosome containing the ADC– antigen complex (Bareford and Swaan, 2007) [15]. An influx of H+ ions into the endosome results in an acidic environment that facilitates the interaction between them Ab component of a fraction of ADCs and human neonatal Fc receptors (FcRns). The bound ADCs are transported outside the cell, where the physiological pH of 7.4 enables the release of the ADC from the FcRn (Roopenian and Akilesh, 2007) [16]. This mechanism acts as a buffer for preventing the death of healthy cells in the case of ADC mis-delivery. Excessive binding of ADCs to tumour cell FcRns might however restrict the release of the cytotoxic drug and prevent the ADC from taking effect (Ritchie *et al*., 2013) [17]. FcRn expression is primarily within the endosomes of endothelial cells. Once the ADC is internalized, the linker and drug have to be released. ADCs that remain in the endosome without binding to FcRn receptors subsequently undergo lysosomal degradation, allowing the release of the cytotoxic drug into the cytoplasm. In ADC the drugs which are more efficiently toxic are used as important component, generally which are not used for chemotherapy (Peters and Brown, 2015) [18].

Conjugate Structure and Desired Characteristics

Monoclonal antibodies are covalently linked to small molecular drugs that target a specific cancer cell and reduce systemic toxicity. Increase the cell-killing potential of monoclonal antibodies (mAbs) and confer higher tumor selectivity. As a result, the tolerability of the drug increases. Compared to standard chemotherapeutic drugs or biologics,

there is limited systemic exposure. An antibody–drug conjugate (ADC) comprises three main structural units: the cytotoxic drug that will be used to kill the targeted cell, antibody (mAb) targeting the specific cell and linkers which joins the antibody and cytotoxic drug by covalent bond.

A. Antibody

Monoclonal antibodies can be generated to recognize and specifically bind to these tumour-associated antigens. Upon binding to the tumor cell, a few "functional antibodies" display modest cell-killing activity by themselves. Antibody discovery was enabled by murine hybridoma technology followed by humanization to deliver therapeutic antibodies with lower risk of immunogenicity (Leung *et al*., 2020) [19]. The production of MAbs by hybridoma technology was discovered in 1975 by Georges Kohler of West Germany and Cesar Milstein of Argentina, who jointly with NielsKaj Jerne of Denmark were awarded the Nobel Prize for Physiology and Medicine in 1984 (Ansar and Ghosh, 2013) [20]. MAbs are comprised of four polypeptide chains, two identical heavy chains and two identical light chains. They are large proteins with total molecular weight of ~150kD. The chains are held together by disulfide linkages and fold to form a "Y" shaped tetramer. With regard to biological function, the amino- and carboxy-terminal halves of an antibody chain are subdivided into variable and constant regions. The variable region shows great variability in the amino acid sequence among antibodies and serves as an antigen binding site, while the constant region determines antibody isotype and effector functions (Moorthy *et al*., 2015) [21].

Antibody selected for inclusion in ADCs depend upon choice of target antigen. MAbs may possess cytotoxic activity by one or more mechanism. IgG1 have ability to mediate CDC (complement-dependent cytotoxicity) and ADCC (antibody-dependent cellular cytotoxicity), while IgG2 and IgG4 Mab are typically deficient in this regard. MAbs allow ADCs to have high target-specificity, target-affinity and prolonged drug exposure at the tumour site. Based on these features, antibody selection should ideally ensure minimal cross reactivity with healthy tissues, sub-nanomolar affinity to the target antigen and a long pharmacokinetic half-life combined with minimal immunogenicity. Overtime, these features result in the accumulation of the ADC at the tumour site and allow it to have an increased therapeutic effect. When constructing the ideal ADC, it is important to maximally preserve the favourable properties of the mAb. The complementarity- determining regions of an antibody (i.e. the antigen-binding sites) should have extremely high (preferably sub- nano molar) target affinity in order to guarantee efficient internalization (Rudnick *et al*., 2011) [22].

The immunogenicity of an ADC is one of the major determinants of its circulatory half-life. Early ADCs made use of murine MAbs that evoked a strong, acute immune response in humans that resulted in the rapid formation of human anti-mouse antibodies within 2 weeks of a single dose (Schroff *et al*., 1985) [23]. Since then, murine MAbs have been replaced with chimeric IgG antibodies that have a human constant region and a murine variable region (Liu *et al*., 1987) [24]. Another alternative is the use of humanized IgG antibodies that have a completely human variable sequence except for the portion responsible for antibody-antigen complementarity (Almagro and Fransson, 2008) [25]. Most ADCs that are currently in use or in clinical development employ either humanized or fully human antibodies (Scott *et al.*, 2012) [26].

B. Linkers

Linkers provide a functional handle for efficient conjugation to antibodies. More sophisticated linkers increase effector solubility, maintain stability in systemic circulation, prevent premature drug release and facilitate the liberation of active drug at the target after internalization. The mechanism of drug release is an important consideration in linker selection. Non-cleavable linkers rely on degradation of the scaffold within the lysosome after internalization. Alternatively, cleavable linkers respond to physiological stimuli such as low pH, high glutathione concentrations and proteolytic cleavage (McCombs and Owen, 2015) [27].

The site of conjugation and choice of linker play a critical role in the stability, the pharmacokinetic properties of ADCs. Attachment sites in antibody MAb can also be engineered via several ways for incorporation of a linker and subsequently the drug. The ideal linker should be stable so that the ADC does not release the cytotoxic drug before reaching its target and causing off-target toxicity. At the same time it should be able to release the drug efficiently once internalized. Based on release mechanism, linkers are generally divided as cleavable and non-cleavable linkers (Dan *et al*., 2018) [28].

1. Cleavable Linkers: The change in environment once the ADC–antigen complex is internalized, triggers cleavage of the linker and release of the active drug, effectively targeting toxicity to cancer cells. Hence cleavable linkers are reliant on distinctive intracellular conditions to release the cytotoxin. There are four types of cleavable linkers: hydrazone, disulfide and peptide linkers, β-glucuronide linker each of which responds to different cancer specific intracellular conditions.

a) **Acid-sensitive linkers (Hydrazone):** Acid-sensitive hydrazone groups in acid-labile linkers remains stable in systemic circulation (pH 7.5) and gets hydrolyzed in lysosomal(pH 4.8) and endosomal (pH 5–6) acidic tumor micro-environment upon internalization in the targeted cells (Pillay *et al.*, 2002) [29]. Withdrawal of Gemtuzumab ozogamicin (Mylotarg®) in 2010, an anti-CD33 ADC for treatment of acute myeloid lymphoma, raises concern over the stability of this linker (Alley *et al*., 2010) [30]. The heterogeneous nature of the drug conjugate contributed to premature release of payload, which in turnmay have contributed to its remarkable toxicity compared to conventional chemotherapy. Currently, inotuzumab ozogamicin and milatuzumab doxorubicin, that are developed with a hydrazone linker.

b) **Glutathione-sensitive disulphide linkers:** Another common example of cleavable linkers is glutathione-sensitive disulfide linkers. Glutathione is a low molecular weight thiol which is present in the cytoplasm (0.5–10 mmol/L) and extracellular environment (2– 20 µmol/L in plasma) (Griffith, 1999) [31]. In tumor cells elevated levels of thiols are found during stress conditions such as hypoxia (Balendiran *et al*., 2004) [32]. The difference in glutathione concentration in cytoplasm and extracellular environment can be implemented as a selective delivery of the drug payload to target tumor via breakdown of disulfide linkers. Maytansinoid drug conjugates have been widely employed for disulfide bonds (Chari, 2008) [33].

c) **Lysosomal protease-sensitive peptide linkers:** The

third type of cleavable linker is the enzyme labile or peptide linker. In comparison with hydrazone and disulfide linkers, peptide linkers offer improved control of drug release by attaching the cytotoxic drug to the MAb via a dipeptide linkage (Sanderson *et al*., 2005) [34].

Tumor cells have higher expression of lysosomal proteases like cathepsin B than normal cells. Cathepsin B-sensitive peptide linker conjugated ADCs selectively binds to and get internalized into tumor cells via receptor mediated endocytosis (Dubowchik and Firestone, 1998) [35]. The proteases required to break the peptide bond are only active in low pH environments, making it highly unlikely that the cytotoxic drug is released in thepH-neutral environment of the blood. Instead, the dipeptide linkage is cleaved in the acidic environment within lysosomes by lysosomal proteases, such as cathepsin-B and plasmin (Koblinsk *et al*., 2000) [36].

Brentuximab vedotin is an example of an ADC that employs a dipeptide linkage consisting of valine and citrulline along with a para-amino benzyl carbamate spacer molecule that separates the large cytotoxic drug from them Mab (Doronina *et al.*, 2008) [37].

d) β-glucuronide linker: β-Glucuronidase sensitive linkers have been successfully used in a handful of glucuronide prodrugs. Lysosomes and tumor necrotic areas are rich in β-glucuronidase which is active at lysosomal pH and inactive at physiological pH (Michelle de *et al*., 2002) [38]. This selective site of action allows for a selective release of cytotoxic payloads through cleavage of the glycosidic bond of β-glucuronidase-sensitive β-glucuronide linkers. Further, the hydrophilic nature of this linker provides aqueous solubility for hydrophobic payloads and decreases aggregation of ADCs (Jeffrey *et al*., 2006) [39].

2. Non-Cleavable Linkers: ADCs with non-cleavable thioether linkers have better plasma stability. Higher plasma stability decreases the non-specific drug release of ADCs as compared to cleavable linkers (Senter *et al*., 2012) [40]. The linker is attached to the amino acid residues of the mAb through a non reducible bond, accounting for high plasma stability. Following internalization, the drug is released from these conjugates due to lysosomal proteolytic degradation of the mAb. The drug-linker-amino acid residue itself must retain the activity of the drug (Diamantis *et al*., 2016) [41]. FDA approved trastuzumab emtansine (Kadcyla®/T-DM1) uses a non-cleavable SMCC (N-succinimidyl-4-(maleimidomethyl) cyclohexane-1-carboxylate) linker to crosslink the warhead DM1 to lysine residues of anti-HER2 mAb trastuzumab. The intercellular metabolite lysine-MCC-DM1 complex was found to be as active as the parent drug, DM1, after lysosomal degradation of trastuzumab (LoRusso *et al.*, 2011) [42].

C. Cytotoxic Drug

To create an effective ADC, it is imperative to have a potent cytotoxic payload. The first generation of ADCs used classical chemotherapy drugs such as doxorubicin and methotrexate with the benefit of a well-known toxicity profile (Shefet-Carasso and Benhar, 2015) [43]. Repeated studies, however, have shown that the actual concentration of the cytotoxic payload in tumor cells is minimal with only 1–2% of the administered dose reaching the tumor. It is evident that the optimal chemotherapy drug used should be extremely potent, being effective at picomolar or nanomolar concentrations.

There are two main categories of cytotoxic drugs used in ADC development: microtubule inhibitors and DNA-damaging drugs. The auristatins, monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF) are specific types of microtubule inhibitors (Francisco *et al*., 2003) [44]. Auristatins block tubulin assembly and cause G2/M phase cell cycle arrest; they are the most commonly used payloads, accounting for a majority of cytotoxic payloads used in ADCs currently investigated (Bouchard *et al*., 2014) [45]. Monomethyl auristatin E, an auristatin derivative (MMAE) is the cytotoxic payload of brentuximab vendotin has a free drug IC50: 10-11 – 10-9 allowing it to be effective in the low nanomolecular range (Gerber *et al*., 2009) [46]. Maytansinoids, another class of tubulin inhibitors, have also been used successfully in the ADC development. The cytotoxic payload of trastuzumab emtansine (T-DM1) DM1 is a highly potent maytansinoid, developed by Immunogen, with a free drug. Tubulysinsare a promising new class of tubulin inhibitors. Tubulisin analogues were successfully conjugated to trastuzumab forming a stable and potent ADC (Cohen *et al*., 2014) [47]. The DNA-damaging like calicheamicins, duocarymycins and PBD dimers are all different types of DNA- damaging agent agents have the ability to be active throughout the different cell cycle phases. Duocarmycin is a powerful cytotoxic alkylating compound that binds to the minor groove of DNA and has shown activity against various multidrug- resistant models. Duocarmycin-based ADCs are currently under investigation in a phase 1 trial setting conjugated to the anti-HER-2 antibody, trastuzumab.

Calicheamicin is a potent antitumour antibiotic that causes double-strand DNA breaks and rapid cell death by binding to the DNA's minor groove. It is less dependent on cell cycle progression making it potentially useful against TICs who have lower rates of proliferation (Sapra *et al*., 2011) [48]. Gemtuzumab ozogamicin and other ADCs such as inotuzumab ozogamicin in non-Hodgkin lymphoma and MDX-1203in renal cancer are using these agents. Pyrrolobenzodiazepines (PBDs) that bind to discrete DNA sequences causing lethal lesions and have interestingly not been found to have cross-resistance with common chemotherapeutic agents (Bouchard *et al.*, 2014) [45].

Lipophilic drugs readily pass cell membranes and therefore have a greater potential to escape the lysosome after release. Conversely, a potential payload must be sufficiently soluble to allow for conjugation to the antibody in aqueous buffers as high concentrations of organic solvent led to antibody scaffold denaturing. The low solubility of many candidate payloads may be balanced by hydrophilic linkers, such as those containing sulfonates or poly (ethylene glycol), allowing for higher DAR than hydrophobic linkers such as SMCC. Acid-sensitive drugs may degrade in the lysosome prior to reaching the site of action; disulfide, alkene-, and epoxide-containing drugs may be reduced or transformed by cellular enzymes. Such drugs must be protected or modified (McCombs and Owen, 2015) [27].

D. Target Antigen Selection

Among the three components of ADCs there is fourth important component i.e target. Indeed, among the four components of ADC only the target is immutable. One may refine and "tweak" the antibody for its affinity, immunogenicity, structure, etc.; the linker for its variable

chemistry, cleavability, etc.; and the drug for its potency and mechanism of action, etc., but the target is determined and controlled by nature. It is beyond the ability of drug developers to tweak and modify the ability of target, so selection of appropriate target should be considered. If an inappropriate target is selected no matter how much time, money and effort should be taken in the development the project is doomed to fail (Bander, 2013) [49].

Aside from being tumour-specific or tumour associated, cell surface antigens should also undergo efficient internalization, have high levels of expression and possess high penetrance. The expression of such antigens should be kept to a minimum on healthy tissue cells (Carter and Senter, 2008) [50]. The target of gemtuzumab ozogamicin, an ADC previously used against Acute myeloid leukaemia, was cluster designation33 (CD33), a transmembrane cell-surface glycoprotein expressed on the surface of mature and immature myeloid cells. CD33 has extremely high penetrance with 90%– 95% of all AML patients testing positive for the antigen (Jilani *et al.*, 2002) [51]. With regard to tumour specificity and sensitivity however, CD33 performed rather poorly as it was found to have only low levels of expression on not only on mature and immature myeloid cells but also erythroid cells, megakaryocytes and multipotent progenitor cells (Ricart, 2011) [52].Current ADCs aim to execute their therapeutic action by identifying target antigens that fulfil all four of their requirements.

There are some target antigen which are present in cancer cells as well as normal cells which include prostate- specific membrane antigen (PSMS) and the HER2 (human epidermal growth factor receptor 2) receptor (Goldmacher and Kovtun, 2011) [53]. In the case of PSMA, it is expressed within the cytoplasm of healthy prostate tissue and therefore remains unaffected by ADCs that target extracellular PSMA in prostate cancer cells. The antigens should be over expressed on cancerous cell for effective targeting.

There is, however, a minimum requirement of approximately 10000 antigens per cell, in order to ensure the delivery of lethal quantities of the cytotoxic drug (Lapusan *et al*., 2012) [54]. Complication arises from the fact that the initial estimate of antigenex pression does not stay constant, but instead varies during the course of the treatment. The rate and efficiency of internalization depends on the type of target and the choice of cytotoxin. Some targets internalize frequently regardless of ligand binding, whereas others reside permanently on the cell surface (Peters and Brown, 2015) [18]. The internalization efficiency of some antigens also depends on the specific epitope that binds to the mAb, as this leads to varying levels of antigen–antibody affinity.

E. Conjugation Between mAb, Linkers and Drug

The heterogeneous nature of drug conjugates and hydrazine linker instability were thought be accountable for the failure of Mylotarg TM. Thus, there was an urgent need for developing new strategies for producing homogenous drug antibody conjugation methods. Side chain cysteine (SH group) and lysine (NH2 group) have been extensively used for conjugation (Table 1). The main problem with these conventional conjugation methods is the heterogeneous nature of the end products with different DAR (drug antibody ratio) values (Hamblett *et al*., 2004) [55]. The conjugation strategy must not alter any key blocks of an antibody that are responsible for its binding to the target antigens

Table 2: Comparison between different side chain conjugation methods.

Conjugation	Reactive groups	advantages
Cysteine residue	Maleimides, haloacetyls, other Michael acceptors	Simple and reproducible method Used in FDA approved Adcetris, widely employed in pipeline candidates, DAR ~0–8Comparatively less heterogeneous by products than lysine conjugation Easier to characterize pharmacokinetically
Lysine residue	Activated ester functional groups like N-hydroxysuccinimide esters	Though highly heterogeneous, this method is employed in FDA approved Kadcyla®, Mylotarg™, DAR ~3.5 (Kadcyla®), ~2.5 (Mylotarg™) Mostly used to crosslink via non- reducible linkers.

1. Via Side chain Cystine Residue: Conjugation via side chain cysteines is a widely utilized and accepted technology in conjugation chemistry of ADCs. Seattle Genetics' ADC brentuximab vedotin utilizes this method to conjugate MMAE with the anti-CD30 mAb (cAC10) via an enzymatically cleavable dipeptide linker (Doronina *et al*., 2003) [56]. Cysteines are engaged in interchain and intrachain disulfide bridges in an antibody, which did not contribute to the building blocks of an antibody. In an IgG1 antibody, there are four interchain disulfide bonds. It was also found that interchain disulfide bonds are more susceptible to reduction than intrachain disulfide bonds, which allow for a controlled reduction of the four interchain disulfide bonds. This can yield upto eight reactive sulfhydryl groups, facilitating drug conjugation with DAR values of 0–8 (Hamblett *et al*., 2004) [55].

These reactive sulfhydryl groups which are nucleophilic in nature, can be reacted with electrophiles like maleimides, haloacetyls for crosslinking proteins (Agarwal and Bertozzi, 2015) [57]. Conjugation via cysteine produces more uniform products than lysine conjugation that are easier to purify and characterize pharmacokinetically.

2. Via Side chain Lysine Residue: Mylotarg™ had utilized side-chain reactive lysine residues of a humanized anti-CD33 mAb for conjugating the drug calicheamicin by a bifunctional acid sensitive hydrazone linker (Hamann *et al*., 2002) [58]. However, Pfizer voluntarily withdrew this product in 2010 (Burnett *et al.*, 2011) [59]. Ado-trastuzumab-emtansine (Kadcyla®), one of four approved ADCs in the market utilizing side chain lysines for conjugating the potent tubulin inhibitor emtansine to mAb trastuzumab (Herceptin®) (LoRusso *et al*., 2011) [42]. An ESI-TOFMS method confirms that 40 out of 86 lysine residues of humanized monoclonalIgG1 huN901- antibody are available for conjugation to DM1 molecules (Luo *et al*., 2016) [60].

3. Drug Antibody Ratio (DAR): DAR is defined as the number of drug molecules per mAb. It determines the dose needed to produce the desired effect in patients. There is a limited number of drug molecules that can be efficiently delivered to the target site and drug loading significantly contributes to the pharmacokinetic profile of ADC. Decreasing the DAR resulted in a superior therapeutic window ofcAC10- MMAE (chimeric monoclonal Ab with

monomethyl auristin E) conjugates, proving that drug loading as a conclusive parameter for designing ADCs. Although cAC10- MMAE conjugates with DAR ~2–4 were less active in *in vitro* studies, but their results in *in vivo* studies were found to be equivalently potent (DAR~4) and better tolerated than the conjugate with higher DAR ~8. Similar observations were found with regards to pharmacokinetic properties (Hamblett *et al*., 2004) [55]. If fewer drug molecules are conjugated per mAb, the ADC system will not be effective clinically. On the other hand, conjugating too many drug molecules per mAb will make the ADC unstable, toxic and may lead to aggregation and immunogenic reactions. Hydrophobic MMAE conjugates using interchain cysteines with higher DAR are found to be physically unstable (Moussa *et al.*, 2016) [61]. ADCs with heavily loaded drugs are more rapidly cleared from the system.In general, an average DAR of 3–4 is used to achieve optimum effect in ADCs, depending upon potency of the payload (McDonagh *et al*., 2006) [2]. Conjugations through side-chain lysine residues are highly heterogeneous leading to inconsistent DAR values and different conjugation sites in the antibody (Dan *et al.*, 2018) [28].

4. Site specific Conjugation: The most common problems with conventional conjugation technologies are heterogeneous by products with different drug distributions per mAb, un-conjugated and overly conjugated mAbs. These phenomena are attributed to poor pharmacokinetic properties and instability of ADCs in systemic circulation (Hamblett *et al*., 2004) [55]. Un-conjugated antibodies occupy the site of attachment, competing with drug-conjugated antibodies and block the site for internalization for the targeting mAb. On the other hand, overly conjugated mAbs are more rapidly cleared as well as can cause immunogenic reactions and toxicity. Engineering of the conjugation site may lead to a more homogenous product with defined and uniform drug stoichiometry. It is done in three ways shown in Table 2.

Table 2: Comparison between different site-specific conjugation technologies.

Method of Conjugation	Reactive group	Advantages	Developer
Engineered side chain cysteine residues (ThioMAb)	Maleimides	Improved clinical safety, tolerability and therapeutic index over conventional conjugates. Controlled and reproducible DAR 2. Compatible for producing in large scale.	Genetech
Incorporation of un-natural amino acids (unAA)	Alkoxy-amine	Highly stable and extended half-life in systemic circulation. Improved pharmacological profile compared to conventional ADCs. Ketone group present in unAA provided conjugation site for different alternative payloads like kinase inhibitors, proteasome inhibitors	Ambrx
Enzymatic Site- Specific Conjugation Process	Amine, Indole	DAR 2-4, More stable conjugates than yielded by Thio MAb and oxime ligation. Controlled conjugation site of the payload on the mAb. Better pharmacokinetic profile over conventional conjugates.	Innate Pharma, Glycos, Pfizer. Inc.

Pharmacokinetic Consideration of ADCS

The need to perform detailed PK studies of both conjugated and released forms of drug after administration of an ADC is critical to fully understand the PK and PD disposition of ADCs. An example of this need was demonstrated by gemtuzumab ozogamicin (Mylotarg®) early trials showed promising remission rates in the high-risk population of older patients with relapsed acute myeloid leukemia (AML). However, confirmatory trials indicated a higher incidence of early fatality in patients receiving gemtuzumab ozogamicin and it was voluntarily withdrawn from the market in 2010. *In vitro* studies showed that the linker was poorly thermostable, with rapid and extensive release of calicheamicin from the antibody, which resulted in toxicity to CD33-negative MOLT-16 cells. This high systemic exposure of the released active drug was implicated in the significant toxicities associated with administration of gemtuzumab ozogamicin in patients (Cianfriglia *et al*., 2013) [62], therefore PK studies are important.

a) Absorption

Physiological barriers limit oral administration of protein-based drugs and needed that most of these agents be administered parenterally to reach systemic circulation (Bruno *et al.*, 2013) [63]. As a result, mAb agents are typically administered either intravenously (iv) or subcutaneously (s/c). For oncology indications, iv infusion is the most frequent route of administration for ADCs with 100% bioavailability. By contrast, there are multiple FDA- approved and commonly used mAbs indicated for inflammatory diseases that are administered via s/c injection, where bioavailability ranges from ~50–80% following s/c administration. However, s/c administration is highly improbable for ADCs, due to the potential reactions to cytotoxic payloads and off-target toxicities mediated by immune cells in the skin, which may cause local deposits of cytotoxic material.

b) Distribution

Due to their size and polarity, the distribution of ADCs is generally restricted to the vascular and interstitial space. Convective transport from blood vessels into tissues is slow and reliant upon pressure gradients. The local structure of both the blood vessel, including fenestration size and membrane thickness, and the surrounding tissue, alters the rate of transport. For example, the tight junctions of blood vessels in the brain effectively limit antibody penetration, resulting in very low distribution of mAbs in brain tissue. By contrast, tumors tend to have leaky vasculature with large pore sizes, allowing increased convective transport of macromolecules into tumors. These are similar barriers that have limited the tumor delivery of other carrier mediated agents, such as nanoparticles and polymer conjugates (Hendry *et al.*, 2016) [64]. However, these tumor barriers may be less of an issue for ADCs, as they are smaller (~10 nm) than nanoparticles (~50–100 nm).

c) Metabolism and Elimination

The metabolism and elimination of mAbs differs significantly from traditional small molecular (SM) drugs. SMs typically undergo renal elimination or phase I and II metabolism, resulting in metabolites with altered polarity, molecular weight, and activity that may be more easily excreted from

the body. Antibody-based therapeutics are cleared via a complex combination of specific and non-specific mechanisms. Degradation of ADCs occurs nonspecifically via proteolysis in a variety of tissues, including the skin, muscle, and liver, due to macrophage uptake (Ferri *et al.*, 2016) [65]. These cells may take up antibodies through non-specific pinocytosis and degrade the engulfed antibodies via lysosomal proteolysis.

Further Development of ADCS for Cancer Treatment

The ADC space continues to develop as knowledge and innovative technologies to improve the therapeutic window of ADCs. Despite the clinical success of Adcetris® and Kadcyla®, the field still faces challenging tasks, such as improving targeted delivery efficiently, minimizing systemic toxicity, and tackling drug resistance (Loganzo *et al.*, 2016) [66]. Incomplete understanding of ADCs mechanism of action, inadequate knowledge of the management and understanding of ADCs off-target toxicities, and difficulties in the selection of suitable clinical settings such as patient selection, dosing regimen are some possible explanations for the slow clinical translation of new ADCs. Their clinical outcomes can be further improved by optimizing target selections, binding moieties (monoclonal antibody and protein scaffolds), cytotoxic drugs, linkers, conjugation sites, and conjugation chemistries. As many novel ADC technologies mature over time, we expect to see a generation of safer and more effective ADCs for clinical translation and commercialization in the future.

Conclusion

The search for "magic bullets" that can potently eradicate cancer without damaging normal tissues continues unabated. Antibody–drug conjugates are one of the fastest growing classes of oncology therapeutics. After half a century of research, the approvals of brentuximab vedotin and trastuzumab emtansine have paved the way for ongoing clinical trials that are evaluating more than 50 further ADC candidates.

Despite its potential, further understanding biochemical, immunological, pharmacological, and molecular aspects of ADCs must be pursued to better design and develop effective ADCs. While choice of target antigens and payloads is important, antibody-payload conjugation methods and linker chemistry are also crucial elements for producing successful ADCs. Instability of the linker and heterogeneity of the product (i.e., broad distribution of DARs) often negatively impacts ADC efficacy and therapeutic window, which often leads to difficulty or limitation in the optimization for clinical application and eventual failure in clinical trials. Further investigations along this line will provide greater insights and sophisticated strategies from medicinal chemistry and pharmacology standpoints, leading to innovative cancer therapeutics in the future.

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