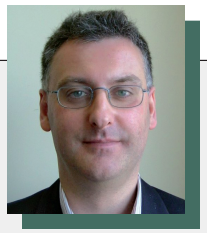

Sean L Kitson

# Antibody-Drug Conjugates (ADCs) – Biotherapeutic bullets

PEER REVIEWED

SEAN L KITSON*, DEREK J QUINN, THOMAS S MOODY, DAVID SPEED, WILLIAM WATTERS, DAVID ROZZELL

*Corresponding author
Almac, Department of Biocatalysis and Isotope Chemistry, 20 Seagoe Industrial Estate, Craigavon, BT63 5QD, United Kingdom

KEYWORDS

Antibody-drug conjugate; ADC; monoclonal antibody; cancer; carbon-14; linker; immunotherapy.

ABSTRACT

Immunotherapies especially targeted towards oncology, based on antibody-drug conjugates (ADCs) have recently been boosted by the US Food and Drug Administration approval of Adcetris to treat Hodgkin's lymphoma and Kadcyla for metastatic breast cancer. The emphasis of this article is to provide an overview of the design of ADCs in order to examine their ability to find and kill tumour cells. A particular focus will be on the relationship between the cytotoxic drug, chemical linker and the type of monoclonal antibody (MAb) used to make up the components of the ADC. Furthermore, the article will conclude on a carbon-14 labelled linker strategy for ADCs to be utilised in absorption, distribution, metabolism and excretion (ADME) studies towards regulatory new drug approval requirements.



## INTRODUCTION

In 2012, the US Food and Drug Administration (FDA) gave approval to 39 drugs and this was the highest number since the mid 1990s (1). According to market analysis, the outlook remains strong for at least 35 new drug approvals per year until 2016 (2). The next blockbuster drug discoveries may well emerge from a class of targeted cell based immunotherapeutics called antibody-drug conjugates (ADCs). These are being developed by biopharmaceutical companies as the next generation of cancer treatments (3).
The first antibody targeted therapy came in 1986 with the FDA approval of OKT3 to treat organ rejection. This involved the use of a murine IgG2a monoclonal antibody (MAb) to target the CD3 antigen, which is a membrane protein on the surface of T-cells (4). Further technologies produced a number of G-type immunoglobulins (IgG) to target oncological conditions: rituximab (chimeric human-murine IgG1 targeting CD20 antigen, non-Hodgkin's lymphoma), trastuzumab (humanized IgG1 targeting HER2 antigen, breast cancer) and alemtuzumab (humanized IgG1 targeting CD52 antigen, chronic lymphocytic leukaemia). Others include bevacizumab (humanized IgG1 targeting VEGF), cetuximab (chimeric human-murine IgG1 targeting EGF receptor) and panitumumab (human IgG2 targeting EGF receptor) for the treatment of metastatic colorectal cancer (5).
This technology of tailoring MAbs has been exploited to develop delivery systems for radionuclides to image and treat a variety of cancers (6). This led to the hypothesis that a cancer patient would first receive a radionuclide antibody capable of imaging the tumour volume. The images of the tumour are obtained by using one or more combinations of the following methods: planar imaging; single photon emission computed tomography (SPECT) and positron emission tomography (PET) (7). These techniques can be extended to hybrid imaging systems incorporating PET (or SPECT) with computed tomography (CT) or magnetic resonance imaging (MRI) (8).

The imaging process is first used to locate the precise position of the tumour and ascertain the appropriate level of the antibody retained by the body. Then the same antibody can be labelled with a radionuclide to deliver a therapeutic radiation dose in order to kill the cancerous cells. This approach has led to radionuclide-antibody conjugates (RACs) being successfully developed to target CD20 antigens for the treatment of non-Hodgkin's lymphoma (NHL). These include ibritumomab tiuxetan (Zevalin) conjugated to indium-111 for imaging and yttrium-90 for therapy approved in 2002 and iodine-131 tositumomab (Bexxar) approved in 2003 (9).
Furthermore, the 'biotherapeutic bullet' approach of ADCs, transporting a cytotoxic drug to the tumour site, to limit the damage to the surrounding healthy cells is an attractive prospect for investors. The market for antibody-based therapeutics continues to expand - especially in the area of oncology - contributing to global revenues of US$50 billion (10). This is due to successful biopharmaceutical partnerships (11) for developing the ADC platform and is demonstrated by numerous clinical trials (12).
The first FDA approved antibody-drug conjugate was Mylotarg (gemtuzumab ozogamicin) in 2000, for the treatment of acute myeloid leukaemia (AML) (13). This ADC targeted the CD33 antigen and a decade later was voluntarily withdrawn from the US market due to a narrow therapeutic window and lack of target-dependence (14). Consequently, this valuable knowledge gained from early ADC development has improved understanding of how to connect antibodies to drugs (15). This technology is producing linkers with an acceptable biological half-life, so that the desired target cell can be reached with limited side effects to the patient (16).

In 2011, the next breakthrough in ADCs came with the accelerated FDA approval of Adcetris (brentuximab vedotin, SGN-35) to treat Hodgkin's lymphoma (HL) and systematic anaplastic large-cell lymphoma (sALCL) (17). Adcetris generated revenues of $34.5 million, for the first quarter of 2012 (18). Another ADC success came in February 2013 when the FDA announced the approval of Kadcyla (trastuzumab emtansine, T-DM1) for the treatment of metastatic breast cancer (19). This ADC combines the blockbuster MAb Herceptin (trastuzumab) with an anti-mitotic maytansinoid drug called DM1 (20). The projected annual sales for this particular ADC are in the region of US$2-5 billion (21).
To date, most of the cytotoxic drugs utilised in the development of ADCs (Table 1) are potent microtubule polymerisation inhibitors (e.g. auristatins, maytansinoids, taxol); or DNA minor groove disruptors (e.g. calicheamicins, duocarmycins) (20) and topoisomerase II inhibitors (doxorubicins and camptothecins). All the above cytotoxic drugs have shown to possess *in vitro* potency against various tumour cell lines in the $10^{-9}$-$10^{-11}$ M range compared to first generation ADCs using doxorubicin ($IC_{50}$ = $10^{-7}$ M) (22). The other emerging drug payloads include the sequence selective DNA alkylating agents called pyrrolobenzodiazepines (PBDs) (23).

## THERAPEUTIC ANTIBODIES

Cancer cells contain a variety of surface antigens that can be utilised as biomarkers to differentiate tumour and non-tumour tissues (24). A number of MAbs have high binding specificity towards tumour-specific antigens. This can induce antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) by lyses of the target cell, resulting in the blocking of signalling pathways (25). Another function of the MAb is to provide a delivery vehicle to transport the drug payload to a surface antigen on the tumour cell, facilitating the internalization of the ADC. This initial work with antibodies displayed high specificities and affinities for receptors that produced an overactive immune response (26). Today biopharmaceutical companies have addressed some of these issues, by modifying the antibodies, to produce a lower immune response in the human body (27).
This lowered immune response is achieved by re-engineering murine antibodies *in vitro,* to allow the part substitution of the murine amino acid sequence for the corresponding human amino acid sequence, to produce chimeric antibodies. These chimeric antibodies are approximately 66% humanized and therefore less likely to trigger an immune reaction. Antibody technology has made it possible to produce high levels of humanized CDR (complementarity determining region) grafted antibodies. Monoclonal IgG antibodies bind to their target antigens through two identical Fab (fragment antigen-binding) arms, each of which includes six CDRs that are a key part of their antigen-binding mechanism (28).
Altering the CDR sequence can increase the specificity and affinity of the antibody. These can be an alternative to chimeric antibodies, which contain 34% mouse protein sequences. A fully humanized antibody protein sequence requires usage of transgenic mice in which murine antibody gene expression is suppressed and replaced with human antibody gene expression. Therefore, engineered antibodies are superior to murine antibodies due to their lower immunogenicity and longer survival duration in the systemic circulation (29).

## MECHANISM OF ACTION OF ADCS

A successful ADC consists of a MAb - a versatile platform for anticancer therapy which is capable of binding to the surface of tumour cell-specific antigens (30). These antigens include over-expressed B-cell surface proteins in non-Hodgkin's lymphoma (NHL) such as CD19, CD20, CD21, CD22, CD40, CD72, CD79b and CD180, extending to the T-cell proteins CD25 and CD30 of the immune system. Moreover, proteins that are over-expressed on carcinoma cells, including the human epidermal growth factor receptor 2 (HER2); prostate-specific membrane antigen (PSMA) and cryptic family protein 1 B (Cripto) are also antigens. These tumour-associated antigens have been studied as potential treatments for the following oncology indications: leukaemia, lymphoma and multiple myeloma (31).
The function of cytotoxic drugs (e.g. auristatins, maytansinoids and calicheamicins), are designed to induce tumour cell death, by causing irreversible DNA damage and/or interfering with the mechanism of cell division (32). The theory behind the mechanism of action of ADCs (Figure 1) involves the following processes: **Binding (Stage 1) -** The MAb component of the ADC binds to the target antigen on the surface of the tumour cell to produce an ADC-antigen (ADC-CDX) complex, which is engulfed into a clathrin-coated vesicle; **Clathrin-Mediated Endocytosis (Stage 2) -** This binding then initiates a cascade of events, involving the internalization of the ADC-antigen clathrin coated vesicle into the tumour cell. Consequently, the vesicle loses its coat and enables the ADC-antigen complex to fuse with an early sorting endosome, to initiate the release of the antigen from the ADC. At this stage, the antigen may be recycled back to the cell membrane. Furthermore, the early endosome converts to a late endosome containing the ADC; **Degradation (Stage 3) -** The internalized ADC is transported through the late endosome pathway to the intracellular compartment of a lysosome, where it is degraded to release the cytotoxic drug. The cleavable linkers rely on processes inside the cell to liberate the cytotoxic drug such as reduction of disulfide bonds mediated by glutathione (GSH) in the cytoplasm, exposure to acidic conditions (pH ~4) in the lysosome, or cleavage by specific proteases within the cell. Conversely, non-cleavable linkers require catabolic degradation (33) of the Mab, to release the cytotoxic drug retaining the linker and amino acid (lysine) residue, by which it was attached to the MAb; **Release (Stage 4) –** The cytotoxic drug enters the cytoplasm, where it binds to its molecular target. In **route A** - calicheamicin based drugs (34) interact with the minor groove of

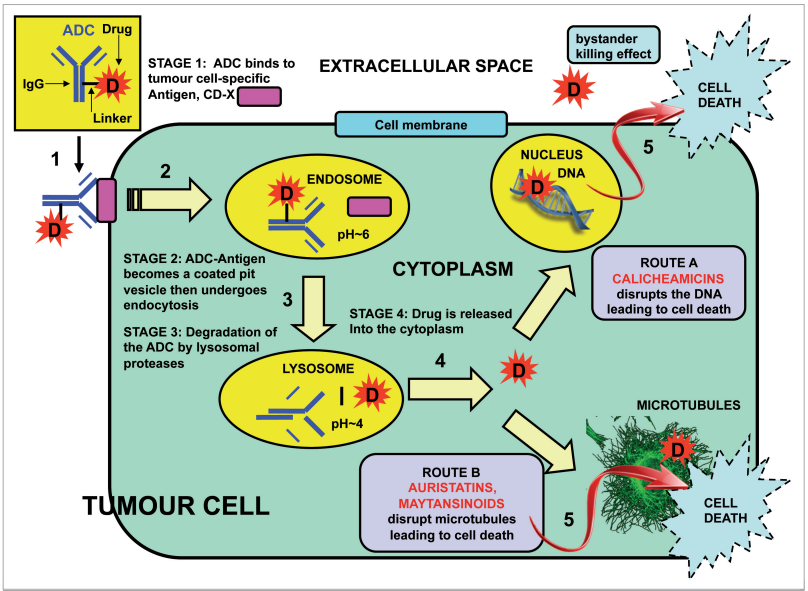


*Figure 1. Schematic representation of the ADC-Antigen internalization process*

DNA and in **route B** – auristatins and maytansinoids disrupt the microtubules (35). Subsequently, the cytotoxic drug may also pass through the cell membrane and enter other cells in close proximity thereby mediating a bystander killing effect; **Stage 5 - Cell Death:** The interaction of the cytotoxic drug with DNA and microtubules initiates a chain of events leading to apoptosis (36).

## ADC DESIGN

The critical factor in the manufacture of ADCs is choosing the right linker to attach the cytotoxic drug to the MAb (37,38). Currently, the development of linker technology is central to biopharma ADC programmes, with the main emphasis on targeting cancer cells. Incorporating the right linker will improve efficacy and reduce detrimental effects, such as the formation of aggregates in the systemic circulation (39). In addition, the linkers can undergo hydrolysis before entering the tumour cell and thereby reducing the potency of the drug (40).
The attachment of the cytotoxic drug to the MAb involves two methods: (**A**) Lysine conjugation occurs when the MAb attaches to the linker to generate the new linker-modified MAb. This linker-modified MAb facilitates a conjugation reaction with the cytotoxic drug to generate the ADC; (**B**) Cysteine conjugation approach involves the inter-chain partial reduction of the disulfide bonds to generate cysteine sulfhydryl (Cys-SH) groups on the MAb. These Cys-SH groups allow for a single step conjugation to the linker-drug to produce the heterogeneous ADC. In both conjugation processes, it is important to control the molar ratios of drug to antibody (DAR) to optimize the *in vivo* pharmacokinetics, efficacy and safety profiles of the ADC (41). Most linkers have a short half-life; for example, the hydrazone-linker in BR96-doxorubicin has an *in vivo* half-life of 43 hours in blood compared to the naked BR96 Mab half-life of several days and/or weeks (38) and therefore chemical modification is required to prolong linker stability and aid solubility (42). The use of non-reducible hydrophilic linkers and/or spacers such as *bis*-maleimido-trioxyethylene glycol (BMPEO) in antibody-linker-drug combinations, has contributed to the biological stability to bypass multi-drug resistance (MDR) of ADCs (43).

The ADC linker platform utilizes **cleavable linkers**: hydrazone and hydrazide moieties; disulfide containing linkers such as *N*-succinimidyl-4-(2-pyridyldithio)pentanoate (SPP) and *N*-succinimidyl-4-(2-pyridyldithio)butyrate (SPDB); 4-(4'-acetylphenoxy)butanoic acid (AcBut) linker; dipeptides valine-citrulline (Val-Cit) and phenylalanine-lysine (Phe-Lys) and **non-cleavable linkers** which include: amide moieties; SMCC (succinimidyl-4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate) and maleimidocaproyl (mc) moiety (44).

The cleavable linkers have a reasonable degree of stability during systemic circulation and can undergo hydrolysis, under certain intracellular conditions such as the acidic environment of the lysosome in the presence of proteases. These disulfide linkers (e.g. SPP and SPDB) are selectively cleaved in the cytosol due to a more reductive intracellular environment (45). Several ADCs (e.g. inotuzumab ozogamicin) have incorporated an acid-cleavable hydrazone linker to enable selective cleavage within the intracellular compartment of lysosomes (pH ~4) during the internalization process.
Another consideration is the use of dipeptide linkers that undergo selective cleavage by lysosomal proteases such as cathepsin B (46). The dipeptide valine-citrulline and phenylalanine-lysine linkers have half-lives of 230 and 80 days respectively in human plasma for the MMAE conjugated to cBR96 MAb. Both linkers can undergo hydrolysis under certain intracellular conditions such as the acidic environment of the lysosome in the presence of proteases (38). Both valine-citrulline and phenylalanine-lysine dipeptide linkers have shown to possess greater serum stability and improved anti-tumour effects compared to the hydrazone conjugate of 5-benzoylvaleric ester of auristatin E (AEVB) (47). Therefore, valine-citrulline (Val-Cit) is suitable for delivery of monomethyl auristatin E (MMAE) to the microtubules. As a result ADCs such as brentuximab vedotin contain the Val-Cit-MMAE linker and exhibit greater *in vitro* specificity and lower *in vivo* toxicity than corresponding hydrazone conjugates (48).
Early development of ADCs focused on linker technology using easily cleavable linkers that guaranteed release of the cytotoxic drug in a timely manner (49). The interest towards non-cleavable linkers led to the development of ADCs that bind with trans-membrane targets. This enabled the internalization of the ADC-antigen complex, whereupon the antibody degrades to release the cytotoxic drug. For example, the formation of a thioether bond between the DM1 maytansinoid and trastuzumab produces trastuzumab emtansine (50).
This thioether linker can replace the Val-Cit dipeptide in ADCs. This enables the ADC to have an extended serum half-life and limits the possibility of early drug release, before it reaches the intended cellular target. Therefore this linker approach removes the uncertainty of labile cleavable linkers (51).

## REGULATORY REQUIREMENTS

In order to release the ADC certain analytical methods must be implemented to verify and identify the type of MAb and cytotoxic drug to be used in its manufacture (52). These analytical techniques are used for the release of the ADC and may include protein mass spectrometry and capillary electrophoresis. A range of analytical tools can be used to determine the molecular weight of the ADC including peptide mapping and sequencing. The structure of the linker-drug combination can further be determined using multi-NMR (53) and FTIR spectroscopy techniques (54). X-Ray crystallography can be used to assess the peptide or antibody structure and the drug to antibody ratio (DAR) can be evaluated using UV methods (55). Subsequently, the application of size-exclusion chromatography (SEC) techniques can be used to determine fragmentation and aggregate patterns during the synthesis of the ADC (56). Furthermore, the antigen binding and biological activity of the MAbs must also be assessed against ELISA, *in vitro* cell-based assays and *in vivo* studies (57). A critical factor is to develop robust analytical methods to determine the level of free cytotoxic drug (58). In addition, chemical impurities obtained during the synthesis which include the impurity profile from host cell proteins must also be identified (59).
The manufactured ADC must be evaluated as a new molecular entity and not as a separate product (antibody-linker-drug) (52). This is to elucidate a structure/function relationship towards: the pharmacokinetics profile and low immunogenicity; the cytotoxic drug must demonstrate potent anti-tumour activity; linker has to be stable to enable the delivery of the ADC to target antigen; MAb must have high affinity and selectivity towards the cellular target. The tumour-associated antigen expression ratio must be high in tumours compared to normal tissue and allow the ADC-antigen complex to be internalized (60).

## ADCS IN CLINICAL DEVELOPMENT

The commercial pipeline of antibody-based biotherapeutics remains strong and now exceeds 350 candidates. Following from the successful FDA approvals of Kadcyla and Adcetris, Table 1 shows 15 ADCs at different stages of clinical development (12, 61).

| Candidate & (Target Antigen) | ANTIBODY-DRUG CONJUGATE [MAb] – [Linker] – [Drug] | Oncology Indication | Developer |
|---|---|---|---|
| **Phase III of Clinical Development** | | | |
| **Inotuzumab ozogamicin** (CD22) | [Hz IgG4] – [Hydrazone] – [Calicheamicin] | NHL | Pfizer |
| **Gemtuzumab ozogamicin** (CD33) | [Hz IgG4] – [Hydrazone] – [Calicheamicin] | Relapsed AML | Pfizer |
| **Phase II of Clinical Development** | | | |
| **Lorvotuzumab mertansine** (CD56) | [Hz IgG1] – [SPP] – [Maytansine DM1] | Solid Tumours, MM | ImmunoGen |
| **Glembatumumab vedotin** (GPNMB) | [Hu IgG2] – [Valine-Citrulline] –[Auristatin MMAE] | Breast Cancer, Melanoma | Celldex Therapeutics |
| **SAR-3419** (CD19) | [Hz IgG1] – [SPDB] – [Maytansine DM4] | NHL | Sanofi |
| **PSMA ADC** (PSMA) | [Hu IgG1] – [Valine-Citrulline] – [Auristatin MMAE] | Prostate Cancer | Progenics |
| **RG7593/DCDT2980S** (CD22) | [Hz IgG1] – [Valine-Citrulline] –[Auristatin MMAE] | NHL | Genentech Roche |
| **RG-7596** (CD79b) | [Hz IgG1] – [Valine-Citrulline] – [Auristatin MMAE] | NHL | Genentech Roche |
| **BT-062** (CD138) | [Ch IgG4] – [SPDB] – [Maytansine DM4] | MM | Biotest |
| **Phase I of Clinical Development** | | | |
| **SGN-75** (CD70) | [Hz IgG1] – [Maleimidocaproyl] – [Auristatin MMAF] | NHL, RCC | Seattle Genetics |
| **BAY 79-4620** (CA-IX) | [Hu IgG1] – [Valine-Citrulline] – [Auristatin MMAE] | Solid Tumours | Bayer |
| **Milatuzumab-doxorubicin** (CD74) | [Hz IgG1] – [Hydrazone] – [Doxorubicin] | MM | Immunomedics |
| **AGS-5ME** (SLC44A4) | [Hu IgG2] – [Valine-Citrulline] – [Auristatin MMAE] | Pancreatic, Prostate Cancer | Astellas |
| **BAY 94-9343** (Mesothelin) | [Hu IgG1] – [SPDB] – [Maytansine DM4] | Solid Tumours | Bayer |
| **ASG-22ME** (Nectin-4) | [Hu IgG1] – [Valine-Citrulline] – [Auristatin MMAE] | Solid Tumours | Astellas |

**Abbreviations** - Ch: chimeric; Hz: humanized; Hu: fully human; MMAE: monomethyl auristatin E; MMAF: monomethyl auristatin F; NHL: non-Hodgkin's Lymphoma; PSMA: Prostate-Specific Membrane Antigen; RCC: Renal Cell Carcinoma; GPMNB: Glycoprotein NMB; AML: Acute Myeloid Leukaemia; MM: Multiple Myeloma; CRC: Colorectal Carcinoma. Source: www.clinicaltrials.gov (12) and adapted from Trail (61).

*Table 1. ADCs currently in clinical development*

## *Brentuximab vedotin*

The antibody-drug conjugate brentuximab vedotin (cAC10-vcMMAE, SGN-35) marketed, as Adcetris (Figure 2) is for the treatment of systemic anaplastic large cell lymphoma (sALCL) and Hodgkin's lymphoma (HL) (62). This ADC uses the chimeric MAb (IgG1 cAC10) to target the human CD30 antigen, (part of the tumour necrosis factor receptor super-family) on sALCL and HL cells. Brentuximab generates its cysteine sulfhydryl (Cys-SH) groups from the mild reduction of the inter-chain hinge disulfide bonds. This facilitates the conjugation of on average 4 drug molecules of monomethyl auristatin E (MMAE, vedotin). MMAE is a synthetic analogue of dolastatin 10, a cytostatic peptide containing several unique amino acid subunits isolated from the marine shell-less mollusk *Dolabella auricularia* (63).

MMAE is so toxic to healthy cells that it cannot be used as a stand-alone chemotherapeutic. Therefore to address this problem, MMAE is conjugated to brentuximab *via* Cys-SH residue by a cleavable linker. This linker combination consists of a thiol-reactive maleimidocaproyl (mc) spacer, the dipeptide valine-citrulline (Val-Cit) linker and a 4-aminobenzylcarbamate (PABC) self-immolative spacer. Under physiological conditions, the linker combination between MMAE and brentuximab remains intact during the rapid internalization of the ADC by the target tumour cell. Consequently, this ADC when incubated with human plasma at 37°C for 10 days indicated less than 2% of unbound MMAE compared to hydrazone-linked conjugates using doxorubicin, which has an *in vivo* half-life of 43 hours in animal models (32).

During the binding process, the ADC-antigen vesicle is internalized by clathrin-mediated endocytosis and transported to the intracellular lysosome compartment. The ADC-antigen complex fuses with the lysosome and the action of cathepsin B proteases initiates a spontaneous intramolecular [1,6]-elimination of PABC to release the free-drug MMAE (potency = $10^{-11}$ – $10^{-9}$ M) into the cytoplasm (64). This drug then inhibits microtubule assembly causing depolymerization leading to cell cycle arrest which results in cell death. Nevertheless, unbound MMAE in the cytoplasm can diffuse through the cell membrane into the microenvironment to exert cytotoxic effects on surrounding cells (65).

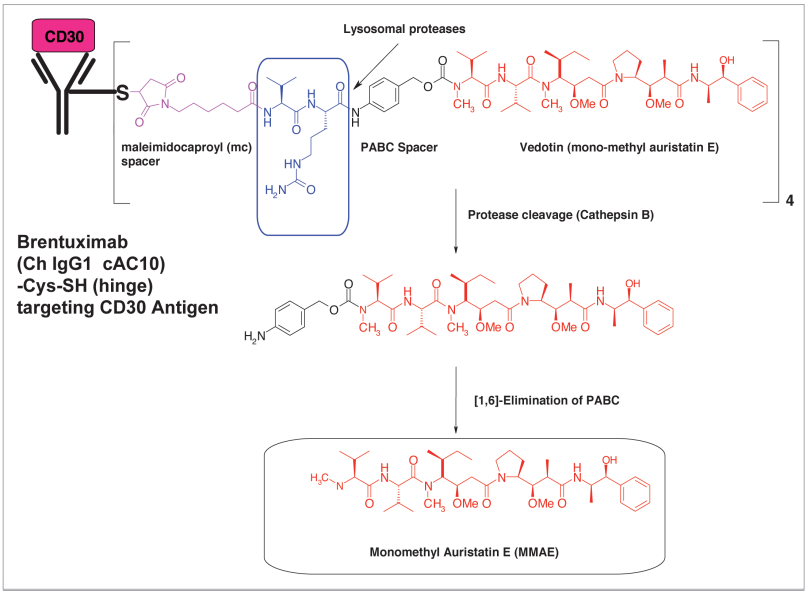


*Figure 2. Schematic diagram of brentuximab vedotin*

### *Trastuzumab emtansine*

Trastuzumab emtansine (T-DM1) marketed as Kadcyla, is the first ADC for treating HER2-positive metastatic breast cancer. This ADC (Figure 3) consists of three components: (**A**) the humanized MAb (IgG1) trastuzumab (Herceptin) to target HER2 tumour antigens; (**B**) the microtubule polymerization inhibitor maytansinoid DM1 drug and (**C**) the (*N*-maleimidomethyl) cyclohexane-1-carboxylate (MCC) linker (66).

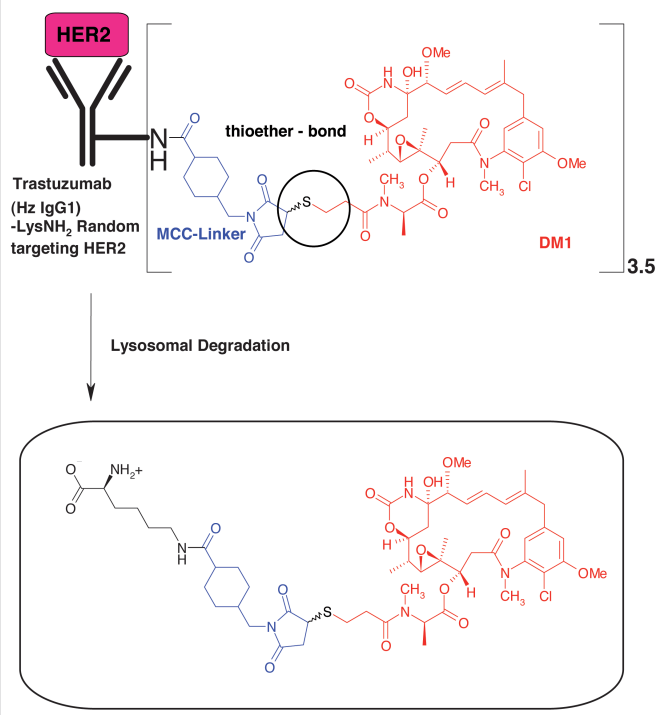


*Figure 3. Schematic diagram of trastuzumab emtansine*

The MAb first undergoes a modification reaction using the non-cleavable heterobifunctional cross-linker succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). This reagent contains *N*-hydroxysuccinimde (NHS) ester and a maleimide reactive group at opposite ends of the cyclohexane spacer arm. The NHS ester reacts with a random surface epsilon-amino group of lysine (1 of 84 available lysine residues) on the MAb to form a stable amide bond, in the linker-modified MAb. The resulting (*N*-maleimidomethyl)cyclohexane-1-carboxylate (MCC) linker containing the terminal maleimide then undergoes a Michael addition with the sulfhydryl (-SH) group of the cytotoxic drug DM1 to form the ADC containing a non-cleavable thioether bond (67).

During preclinical development of this ADC, several other linkers were tested and all containing disulfide bonds (68). These disulfide moieties were susceptible to *in vivo* reduction, which resulted in the early release of the maytansinoid and the Mab into the systemic circulation.

Fortunately, this MCC linker forms a thioether bond instead of a disulfide bond and therefore cannot undergo cleavage by reduction within the cell. The ADC undergoes catabolic metabolism within the tumour cell to release the cytotoxic drug DM1 from the MAb, thereby retaining the linker and gaining a lysine residue. Trastuzumab emtansine produces a better efficacy and pharmacokinetic profile with less toxicity than conjugates developed with other linkers (69).

The majority of ADCs contain a number of the same drug attached to the Mab, thereby producing heterogeneous mixtures. Trastuzumab emtansine exists in such a heterogeneous form ranging from 0-9 DM1 drug-molecules on each antibody with an average of 3.5 DM1 molecules per antibody (39). The tumour killing action of DM1 is in the inhibition of cell division by binding tubulin, arresting the target cell in the G2/M stage of the cell cycle resulting in apoptosis (70).

### *Inotuzumab ozogamicin (CMC-544)*

The ADC inotuzumab ozogamicin (CMC-544) (Figure 4) is undergoing numerous clinical trials. These include Phase III trials in patients with relapsed/refractory acute lymphoblastic leukaemia (NCT01564784) and in combination with the anti-CD20 antibody rituximab in patients with relapsed/refractory aggressive non-Hodgkin's lymphoma (NCT01232556) (12).

CMC-544 contains the humanized, hinge-stabilized (IgG4 SerPro G5/44) MAb directed against the CD22 antigen found on mature B cells. This MAb is covalently linked through an epsilon-amino group of lysine to the DNA-disrupting pro-drug *N*-acetyl-γ-calicheamicin dimethyl hydrazide (CalichDMH) *via* an acid-labile 4-(4'-acetylphenoxy)butanoic acid (AcBut) linker. The resulting ADC is stabilized by the geminal methyl groups adjacent to the disulfide linkage to prevent the early release of CalichDMH and the components of this ADC resemble that of Mylotarg (71).

Mylotarg (gemtuzumab ozogamicin) contains the humanized (Hp67.6 IgG4) MAb targeting the CD33 antigen, using the AcBut linker and CalichDMH as in CMC-544. In addition, Mylotarg is a heterogeneous mixture of 50% conjugates containing 0 to 8 calicheamicin moieties per gemtuzumab. Moreover, CMC-544 has shown to be more effective *in vitro* than Mylotarg for killing acute lymphoblastic leukaemia cells. In spite of the high potency ($10^{-10}$ – $10^{-9}$ M) of the calicheamicin moiety, initial clinical trials suggest using low doses of the CMC-544 (72).

This calicheamicin toxin, used in both ADCs contains the enediyne antibiotic moiety, which comes from the bacterium *Micromonospora echinospora* producing calicheamicin-γ1 (73) and was first isolated in the 1980s. These potent calicheamicin toxins work by destroying the

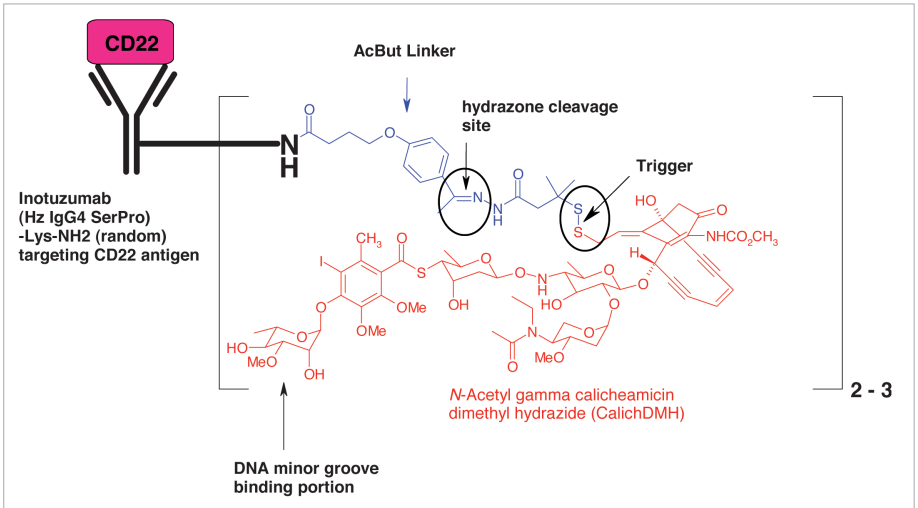


*Figure 4. Schematic diagram of inotuzumab ozogamicin*

DNA of cancer cells (74). Furthermore, inotuzumab has a much higher affinity for the CD22 antigen, resulting in an increased rate of internalization of the ADC-antigen complex, in comparison with Mylotarg (75).
The mechanism of action of calicheamicin (Figure 5) containing ADCs act by cutting the DNA using the enediyne aglycone functionality, otherwise known as the therapeutic bullet (76). These calicheamicin toxins contain an aryl *tetras*accharide, which bind to DNA in the minor groove to the following sequence d(TCCT)-d(AGGA) which is associated with carbohydrate-DNA recognition (77). Upon rapid internalization, the ADC-antigen vesicle is transported to the lysosome intercellular compartment. There, under acidic conditions (pH~4), the acid labile hydrazone functional group within the AcBut linker is hydrolyzed allowing for the release of calichDMH. This resultant pro-drug diffuses into the nucleus, where it binds DNA in the minor groove resulting in reductive cleavage of the disulfide bond which is mediated by glutathione. This triggers spontaneous changes in the enediyne moiety, which generate reactive *para*-phenylene diradical species. These remove hydrogen atoms from the phosphodiester carbohydrate backbone of the DNA producing double-strand DNA breaks, leading to cell death (78).

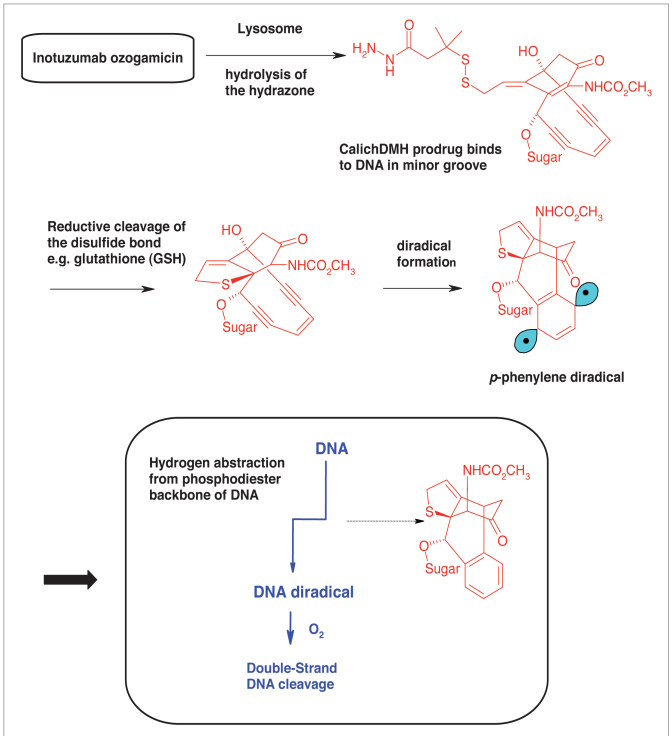


*Figure 5. DNA cleavage by CalichDMH*

## CARBON-14 LABELLING OF ADCS

The number of antibody biotherapeutics in preclinical and clinical development is increasing and therefore the need for evaluation of the pharmacokinetic profile of these ADCs is becoming more important (79, 80).
Notwithstanding advancement of analytical technologies, radiolabelling of drugs remains the gold standard to facilitate absorption, distribution, metabolism, and excretion (ADME) studies (81).
Biopharmaceutical companies need a reliable supply of isotopically labelled active pharmaceutical ingredients (APIs) (82). The isotope of choice is carbon-14 to produce API for Phase 0 to Phase III; ADME; mass balance (83) and micro-dosing studies (84). For human studies, this necessitates procedures to perform synthesis and repurifications under cGMP conditions, in compliance with ICH Q7A Section 19 guidelines (single batches for investigational drugs) and/or MHRA guidelines for the preparation of carbon-14 labelled APIs for clinical trials (85). These carbon-14 labelled compounds provide vital information for chemistry, manufacturing and controls (CMC) towards investigational medicinal product dossier (IMPD) submission (86).
The major advantage of radiolabelling with carbon-14 is that it can be incorporated into the drugs' carbon framework without altering its chemical structure, thereby producing an identical copy of the unlabelled drug. This approach reduces the risk of the carbon-14 label being scrambled, in comparison to 'peripheral' tritium labelling (87). Consequently, during the design of the synthetic route, it is vital to locate a feasible, biologically stable position for the carbon-14 label and to identify suitable starting materials which can be commercially available or easily made (88).

Carbon-14 labelling of ADCs can be executed on the linker part or incorporated into the cytotoxic drug or both. These cytotoxic drug families involving auristatins, maytansinoids and calicheamicins have complicated chemical structures containing several chiral centres (89).
In these cases, carbon-14 labelling is performed on the linker part of the ADC component. Ideally, the label should be placed in the most metabolically stable position on the linker to be able to survive in the systemic circulation before internalization of the ADC-antigen complex into the tumour cell (90).

This strategy is demonstrated in the radiolabelling of trastuzumab emtansine (Figure 6) and utilises the carbon-14 labelled SMCC linker (66).

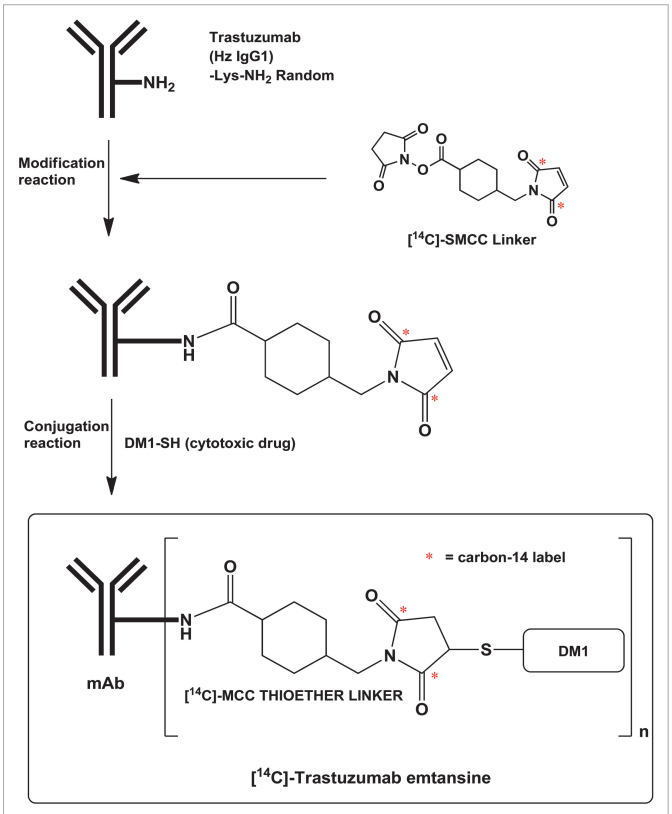


*Figure 6. Carbon-14 labelling sites in trastuzumab emtansine*

The NHS-ester of [$^{14}$C]-SMCC reacts with a random surface epsilon-amino group of lysine on the MAb to form a stable amide bond in the [$^{14}$C]-linker-modified MAb (67). Carbon-14 labelled SMCC can be synthesized from [1,4-$^{14}$C]-maleic acid and 4-aminomethylcyclohexane-1-carboxylic acid (91). The terminal [$^{14}$C]-maleimide moiety in SMCC can then undertake a conjugation reaction with the cytotoxic drug DM1. This produces a non-reducible thioether linker and the ADC contains two carbon-14 labels allowing for a maximum specific activity of 120 mCi/mmol per drug molecule (91). An alternative strategy would be to apply tritium labelling on the methoxy moiety of the aromatic ring in DM1 (92).

The carbon-14 labelled ADC then undergoes a purification process using a system which facilitates ultra-filtration and diafiltration (93). A critical function of this system is to remove unbound [$^{14}$C]-linker and/or cytotoxic drug to ease purification using hydrophobic interaction chromatography (HIC). This enables the isolation of carbon-14 labelled ADC, containing no labile cytotoxic drug (94). The final product purity of the carbon-14 labelled ADC can be determined using size-exclusion chromatography (SEC) analysis (55).

Furthermore, carbon-14 labelled ADCs can be used to determine mass balance, routes of elimination, identify circulatory and excretory metabolites and determine clearance mechanisms (81, 83). Other functions include identification of parent compound and its metabolites, validation of preclinical models and MIST (metabolites in safety testing) (95, 96). Finally, carbon-14 labelled ADCs can be utilised for first-in-human studies using accelerator mass spectrometry (AMS) to obtain pharmacokinetics and metabolic profiles of radiolabelled ADCs (97).



## CONCLUSION

The concept of targeted therapy towards the treatment of disease causing agent was first postulated by Paul Ehrlich over 100 years ago (98). The idea was to create the ideal therapeutic agent, termed the chemical 'magic bullet,' to attack specific cellular targets, in the fight against disease states.
Ehrlich's vision is now being realized for cancer treatment with the development of targeted therapies using MAbs with peptides and/or proteins. The production of rodent MAbs using hybridoma technology first developed by Milstein and Köhler (99) in 1975, led to antibodies having a single specificity towards the cognate antigen. This technology platform of tailoring the MAbs has been exploited by numerous biopharmaceutical companies to develop delivery vehicles for radionuclides and drug payloads to image and treat a variety of cancers.
The emergence of the new class of ADC chemotherapeutics is able to deliver a drug payload to its tumour target, guided by a specific MAb. This is an exciting opportunity for biopharmaceutical companies and contract manufacturing organizations (CMOs) to contribute towards the next generation of ADCs. These biotherapeutic advances for the development of future drugs in their various clinical stages, will bring many regulatory issues to the forefront regarding the ADME profile of each ADC.
This requirement for carbon-14 labelling of the synthetic chemical linker and/or the drug payload will be able to identify potential metabolite(s) in the systemic circulation of various animal models. Furthermore, carbon-14 labelled ADCs may also be utilised in human micro-dosing AME (absorption-metabolism-excretion) studies in order to obtain critical information on cellular targets towards FDA drug approval. Ultimately, ADC therapies will facilitate personalized medicine for the treatment of oncology disease states.

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