Module code: S390 **Name: Chloe Wright**

Assignment: EMA

Antibody-drug-conjugates: The recent developments and Trastuzumab deruxtecan in the treatment of HER2 breast cancer.

A report submitted as the examined component of the project module SXC390.

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Drug Design and Synthesis

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Abstract

Cancer poses a significant risk to many people with 11.7% of all those diagnosed with cancer in 2020 being female breast cancer. Previously approved drugs to treat cancer have proven to be too potent and exert toxic side effects. The aim of this review is to evaluate trastuzumab deruxtecan in the treatment of breast cancer.

A review of current scientific literature on trastuzumab deruxtecan and antibody-drug conjugates comprising of primary research, review papers and other sources forms the basis of this project. The scientific literature has been identified using major scientific databases, search engines and other sources. Key words used for the searches include *antibody-drug conjugates, trastuzumab deruxtecan, mode of action* or *mechanism, synthesis, recent advances* and *exatecan.*

HER2 receptors are overexpressed in those found to have HER2 positive breast cancer. This is targeted by trastuzumab deruxtecan whose monoclonal antibody, trastuzumab, binds to HER2 receptors. The cytotoxic payload released within the cell, DXd, acts as an inhibitor binding at the interface of the topoisomerase I-DNA complex. This leads to an accumulation of cleaved DNA due to inhibition of unwinding and reannealing of the double helices and subsequently cell apoptosis.

The synthesis of trastuzumab deruxtecan is challenging and takes place through multiple steps however several recent improvements have been made. These include late stage coupling of exatecan and the chemical drug linker.

Clinical benefits exerted by trastuzumab deruxtecan due to its high drug to antibody ratio has led to further research into developing antibody-drug conjugates with high drug to antibody ratios. This report reviews these recent developments and suggests areas for future research.

Trastuzumab deruxtecan has been given full approval to treat patients with HER2 positive breast cancer which is either metastatic or cannot be removed by means of surgery. It remains as one of thirteen currently approved antibody-drug conjugates.

300 words

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Abbreviations

ADC	Antibody-drug conjugate
Boc-GGFG-OH	Tert-butoxycarbonyl glycyl glycyl phenylalanyl glycine
DAR	Drug to Antibody Ratio
DBU	1,8-Diazabicyclo
DIPEA	Diisopropylethylaminews
DMA	Dimethylacetamide
DME	Dimethoxyethane
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDCI	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
GGFG	Glycine-glycine-phenylalanine-glycine
FDA	Food and Drug Administration
HER	Human epidermal growth factor receptor
HER2	Human epidermal growth factor receptor 2
HBV	Hepatitis B virus
HCl	Hydrochloric acid
HIV	Human immunodeficiency viruses
IM-ADC	Immune modulating antibody-drug conjugate
mAb	Monoclonal Antibody
mc-Osu	6-maleimidohexanoic acid N-hydroxysuccinimide ester
MMAE	Monomethyl auristatin E
MMAF	Monomethyl auristatin F
MOA	Mechanism of action
MsOH	Methanesulfonic acid
NBS	N-Bromosuccinimide

NK	Natural Killer
***o*-cresol**	Ortho-cresol
PBD	Pyrrolobenzodiazepine dimer
PNU-159682	Iodoacetamide reagent
PPTS	Pyridinium p-toluenesulfonate
PSAR	Polysarcosine
RNA	Ribonucleic acid
rt	Room temperature
TCEP	Tris (2-carboxyethyl)phosphine
T-DXd	Trastuzumab Deruxtecan
TEA	Triethylamine
TFA	Trifluoroacetic acid
TFAA	Trifluoroacetic anhydride
THF	Tetrahydrofuran
TKM	Tandem Knoevenagel-Micheal addition
UDP-Gal	Uridine diphosphate galactose
WSCI	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride

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Chapter 1

Introduction

1.1 – Background

Breast cancer can be identified as the most common cancer women around the world are diagnosed with (Tiersten and Goldman, 2020) in 2020 11.7% of all cancer diagnoses were female breast cancer (Sung *et al*., 2021).

Since the early 1970s medical advancements in less toxic and invasive breast cancer treatments resulted in a 41% decrease in breast cancer mortality rates (Cancer Research UK, 2019). Although improvements have been made, throughout 2020 2.26 million women were diagnosed with breast cancer and 685,000 deaths were reported globally (BCUK, 2022).

Drugs have been trialled in the treatment of cancer for decades however limitations including off-target specificity result in toxic side effects. To overcome limitations studies developing a highly potent biopharmaceutical drug, antibody-drug conjugates (ADC), began and were successful in 1983 (Nawrat, 2021). To date there are only thirteen ADCs currently approved by the Food and Drug Administration (FDA) (Axispharm, 2023). The first ADC, gemtuzumab ozogamicin, was approved in 2000, this was later withdrawn due to safety concerns and lack of evidence to support claims of its clinical benefits (Nakada *et al*., 2016).

As of March 2019, Daiichi Sankyo and AstraZeneca confirmed a global collaboration to develop and commercialise trastuzumab deruxtecan (T-DXd) in the treatment of human epidermal growth factor receptor 2 (HER2) positive breast cancer which is either metastatic or cannot be removed by surgery (AstraZeneca, 2019). T-DXd was the second ADC to be approved by the FDA containing the antibody trastuzumab (Axispharm, 2023) and has shown outstanding clinical results due to its high drug to antibody ratio (DAR) and potent mechanism of action (MOA).

1.2 – Objectives

This project will:

- describe the disease T-DXd treats and the extent to which the disease affects the world population;
- define and illustrate the structure of T-DXd and the benefits of its structure;
- review the mechanism of action and existing synthetic routes of T-DXd;
- review recent advances in the chemistry of ADCs;
- discuss strengths and weaknesses of ADCs and suggest suitable future recommendations.

1.3 – Scope

The project comprises of a critical evaluation and review of the recent scientific literature regarding T-DXd and ADCs. This project investigates research into the mechanism of action of T-DXd in targeting HER2 breast cancer and synthetic routes to improve production for clinical use. An aim of this review is to identify potential future areas of research.

1.4 – Methodology

The main basis of this project comprises primary research papers with a limited number of review papers and other sources. Papers have been sourced through search engines or scientific databases including Web of Science, the Royal Society of Chemistry, Scopus, and Google Scholar. Key words used to complete literature searches include or combine *antibody-drug conjugates, trastuzumab deruxtecan, mode of action* or *mechanism, synthesis, recent advances* and *exatecan*.

Chapter 2

Structure

2.1 – General structure of antibody-drug conjugates

ADCs have a specific structure which has been studied and designed to overcome the toxic side effects caused previously by cancer drugs. Their structure enhances selectively ensuring only tumour cells are targeted on administration, minimizing toxicity. ADCs are constructed from a monoclonal antibody (mAb) (fig. 1) which aids selectivity by targeting antigen receptors found on the exterior of tumour cells. They also contain a cytotoxic payload (drug) responsible for cell apoptosis of the tumour cell once identified by the mAb (Li *et al*., 2019). The cytotoxic payload present within ADCs is often highly potent due to an only a small number of ADCs reaching the target cells. The mAb and cytotoxic payload are covalently bound (Deslignière *et al*., 2022) together by a chemical linker responsible for joining the two components. The linker must be highly stable ensuring degradation of the ADC and release of the cytotoxic payload only occurs within the tumour cells.

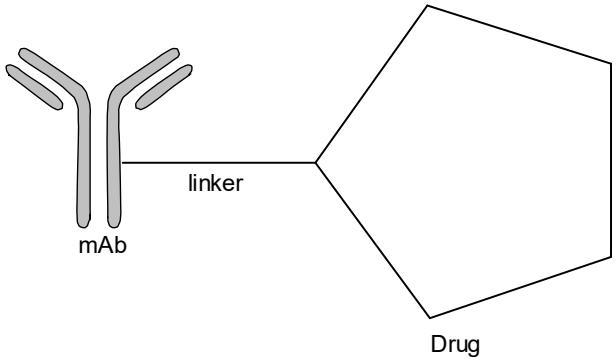


Figure 1 – The general structure of antibody-drug conjugates.

2.2 – Structure of Trastuzumab deruxtecan

T-DXd has been designed with the aim to treat HER2 positive breast cancer therefore the three individual components must be suitable for the chemical environment they are required to reach. T-DXd consists of a mAb based on the amino acid sequence of trastuzumab (Husna and Wong, 2022), which targets HER2 receptors, a glycine-glycine-phenylalanine-glycine (GGFG) enzyme cleavable linker (Nakada *et al*., 2016) and the cytotoxic payload DXd, a topoisomerase I inhibitor (Deslignière *et al*., 2022) (fig. 2). T-DXd has a high DAR of eight therefore for every molecule of trastuzumab eight DXd molecules are present (Sheikh and Huang, 2023), this was supported by hydrophobic interaction chromatography investigations completed by Deslignière *et al*. (2022).

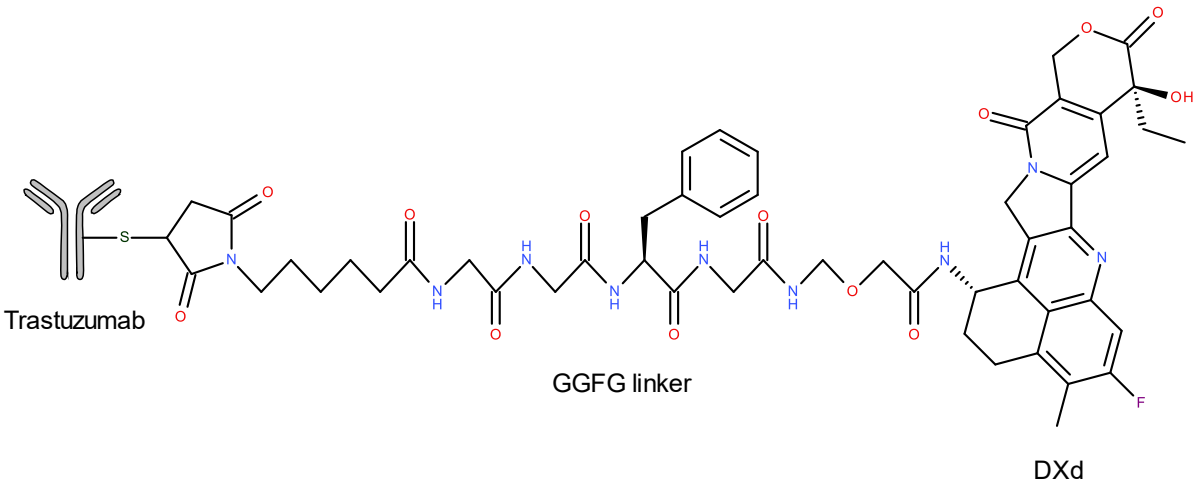


Figure 2 – The structure of T-DXd an antibody-drug conjugate.

DXd is a derivative of exatecan therefore bearing similarities in their chemical structure. The variations in structure are present on the nitrogen bearing group covalently bound to the cyclic ring, DXd contains an amide and alcohol group whereas exatecan contains an amine group (fig. 3). Both DXd and exatecan are also camptothecin derivatives, camptothecin is a metabolite isolated from the Chinese ornamental tree *Camptotheca acuminata* Decne (Pu *et al*., 2019). Camptothecin faces many challenges such as low drug loading efficiency, poor cellular uptake and premature drug release therefore extensive research has been completed to develop derivatives such as DXd in aims to reduce their side effects and improve efficacy (Wong *et al*., 2023).

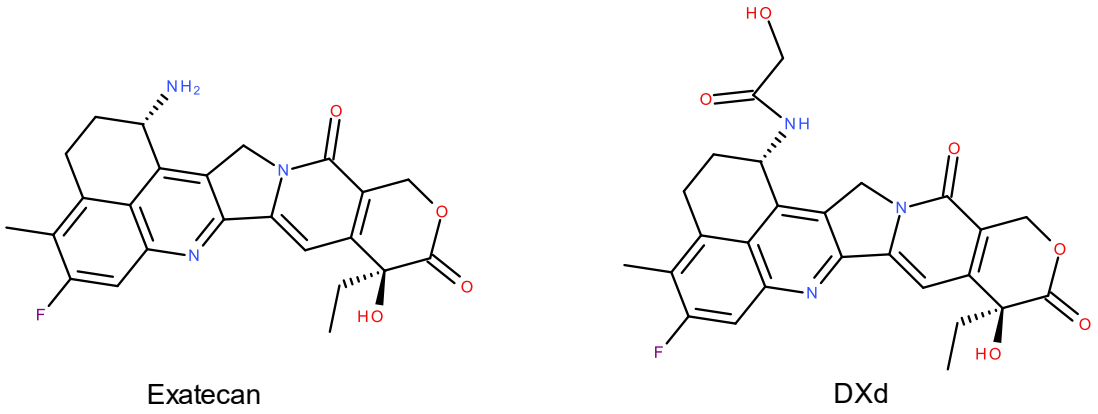


Figure 3 – The structure of exatecan and DXd an exatecan derivative.

2.3 – Recent advances in antibody-drug conjugate structure

Although the structure of ADCs has resulted in successful trials research continues to improve their structure and extend their capabilities. The hydrophobicity of ADCs contributes to their physicochemical properties with studies showing an increase in hydrophobicity correlates to improved *in vitro* potency (Buecheler *et al*., 2018). An increase in hydrophobicity of the ADC may also lead to poor solubility in water and decreased metabolic stability leading to potential nonspecific toxic side effects (Buecheler *et al*., 2018). ADCs with a higher DAR have been found to have increased hydrophobicity due to the increased number of hydrophobic drug molecules bound within the conjugate. This discovery often limits DAR values of ADCs to between two and four (Viricel *et al*.,

2019). Due to these limitations drug structure must be studied extensively to take advantage of increased hydrophobicity whilst minimising the side effects.

Viricel *et al*. (2019) reported the production of a polysarcosine (PSAR) drug linker capable of masking the hydrophobicity of the cytotoxic payload overcoming the limitations of ADCs with high DARs. This research group initially completed their investigations with the aims to mask the hydrophobicity of the payload monomethyl auristatin E (MMAE) (fig. 4), however more recent studies completed by the group investigated the implementation of the PSAR drug linker to mask the hydrophobicity of exatecan (Conilh *et al*., 2021). Both studies completed by the research group found that the PSAR drug linker masked the hydrophobicity of the payloads improving DAR whilst maintaining physiochemical and pharmacological properties. The exatecan based ADC developed by Conilh *et al.* (2021) also saw anti-tumour properties which exceeded the performance of T-DXd.

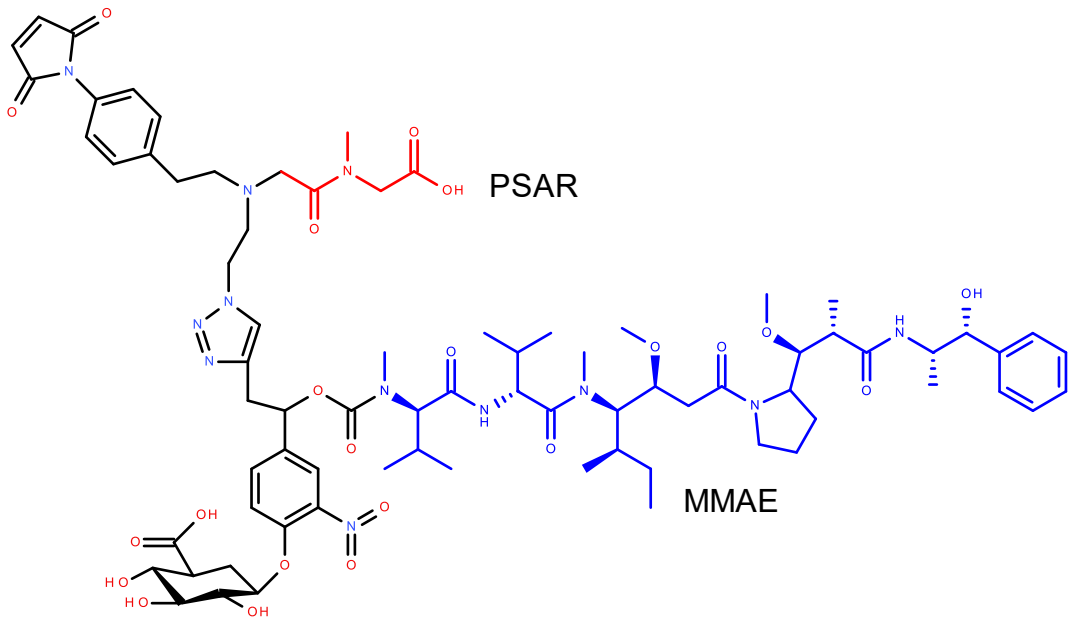


Figure 4 – An image to show a PSAR based drug linker ADC. Adapted from Viricel *et al*. (2019).

In an aim to increase the DAR of ADCs researchers have also developed ADCs which can undergo dual site-specific conjugation. Zhang *et al*. (2021) have produced an ADC utilizing trastuzumab, the anti-HER2 antibody, to produce a dual payload conjugate with site-specifically engineered p-acetyl-phenylalanine and cysteine. The two mechanistically different cytotoxic drug will bind to different areas of the mAb due to the binding at different functional groups present (Zhang *et al*., 2021).

2.4 – Where next

T-DXd has demonstrated the advantages ADCs with high DARs can possess therefore future research should maximise these findings. Although high DAR ADCs show promising clinical results issues with hydrophobicity must be considered due to its potential to decrease metabolic stability and water solubility (Buecheler *et al*., 2018). Viricel *et al*. (2019) found promising results with the production of a PSAR drug linker which could mask the hydrophobicity of the ADCs. Future studies should determine the drug linker's ability to mask hydrophobicity when implemented into alternate ADCs and its potential to mask the hydrophobicity of dual payload ADCs produced by Zhang *et al*. (2021).

Future research should also determine if alternate linker structures can be developed to improve hydrophobicity masking.

All components used to produce ADCs play a key role in the delivery of the cytotoxic payload to tumour cells however the linker ensures the cytotoxic payload is not released prior to reaching the tumour cells preventing toxic side effects (Su *et al*., 2021). Due to its significance the most suitable linker must be determined for each ADC therefore further research should be completed to ensure during future development of ADCs the most efficient linker is selected minimizing chances of early drug release.

Chapter 3

Mechanism of action

3.1 – Endocytosis

The MOA of T-Dxd remains unclear however various scientific research papers support each other's findings and discuss the mechanisms detailed below.

The biological target for T-DXd is the extracellular domain IV of the HER2 antigen present on HER2 positive cancer cells. HER2 is found to be overexpressed in 20-30% of patients diagnosed with breast cancer (Zhao *et al*., 2021). HER2 is part of the human epidermal growth factor receptor (HER) family and is responsible for controlling cell growth and differentiation. The structure of HER2 receptors comprises a cysteine rich extracellular binding site for ligands and an intracellular domain for tyrosine kinase catalytic activity (Yamaoka *et al*., 2018).

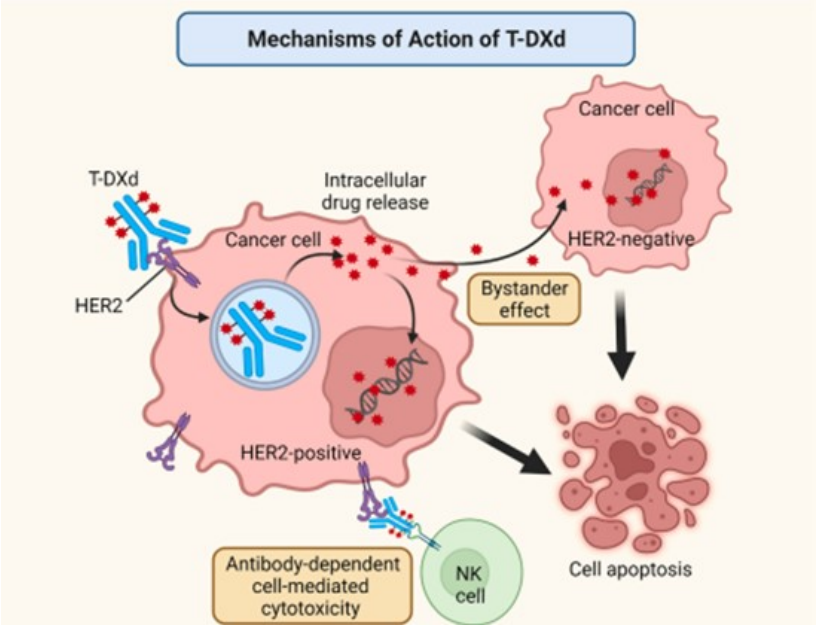


Figure 5 – A depiction of the mechanism of action of T-DXd (Husna and Wong, 2022). Used with permission of Elsevier Science & Technology Journals, conveyed through Copyright Clearance Center, Inc.

Trastuzumab the anti-HER2 mAb binds to HER2 antigens on the HER2 positive cells surface (fig. 5), using intermolecular forces such as hydrogen bonds. Binding initiates receptor-mediated internalization of T-DXd into the cell. Once internalized lysosomal enzymes within the cell cleave T-DXd breaking the peptide bond between the cytotoxic payload and linker (Husna and Wong, 2022) followed by concomitant amine immolation releasing DXd (Li *et al*., 2019) (fig. 6). Research by Ogitani *et al*. (2016) and Sheikh and Huang (2023) supports the theory of DXd releasing as the cytotoxic payload after lysosomal degradation.

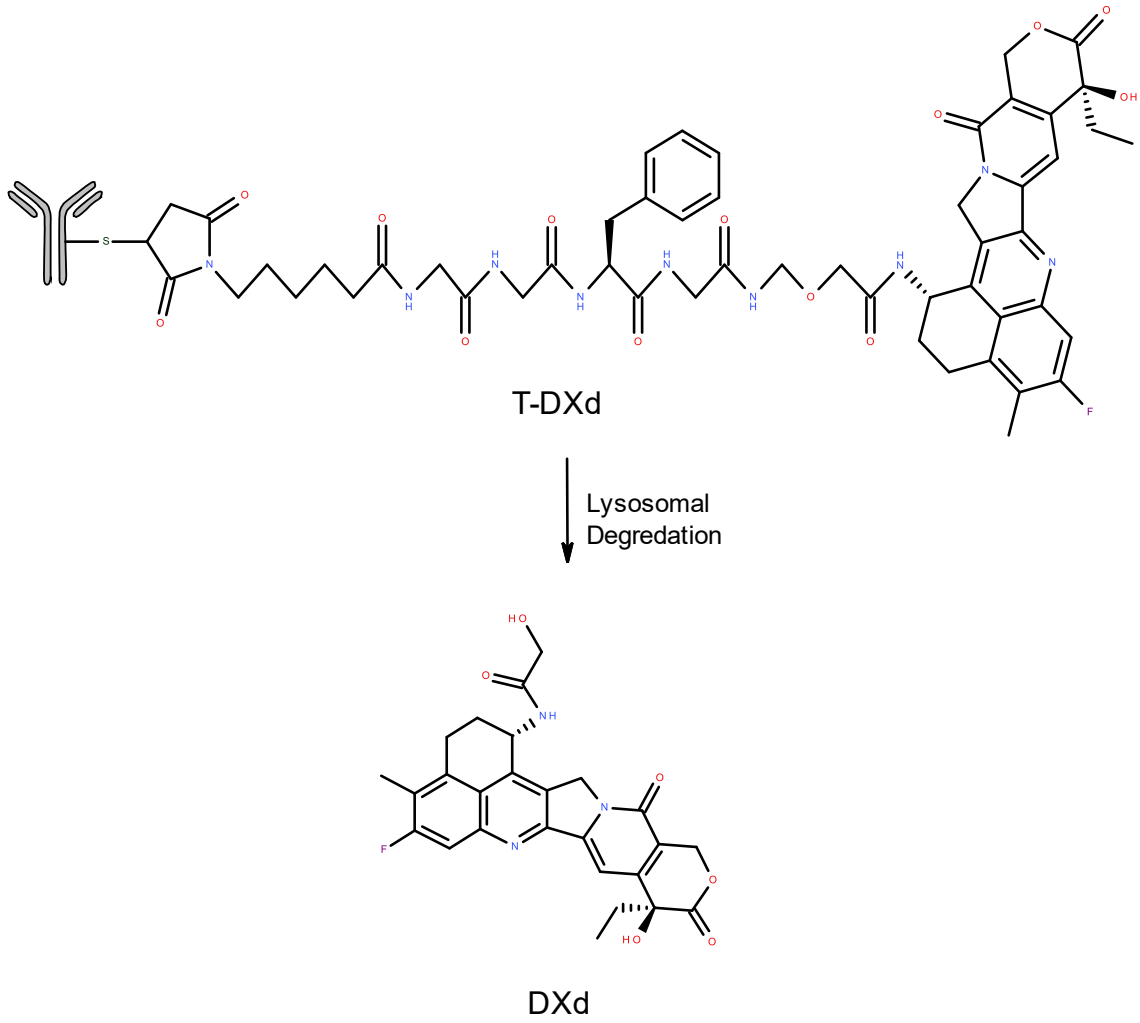


Figure 6 – A figure to show the lysosomal degradation of T-DXd releasing DXd.

DXd is an exatecan derivative (fig. 3) with the ability to stimulate cell apoptosis, DXd will target topoisomerase I enzyme when bound to deoxyribonucleic acid (DNA) as the topoisomerase I-DNA complex. The topoisomerase I enzyme within the cell takes part in DNA replication and ribonucleic acid (RNA) synthesis. Topoisomerase I is responsible for cleaving a single strand of DNA, partially unwinding, and reannealing the strand relieving tension during DNA replication (Li *et al*., 2019). Inhibition of this process by DXd will prevent reannealing of the DNA strand resulting in an accumulation of cleaved DNA within the cell and consequently cell apoptosis.

Although studies have not investigated the binding of DXd to the topoisomerase I-DNA complex recent studies have investigated how camptothecin and its derivative exatecan bind to the topoisomerase I-DNA complex after degradation. Studies found that camptothecin and its derivatives act as interfacial inhibitors, binding at the interface of the topoisomerase I-DNA complex cleavage site through hydrogen bonds and π- π stacking. Hydrogen bonds will form between the cytotoxic payload and topoisomerase I amino acid residues and π- π stacking bonds will be formed with the base pairs adjacent to DNA (fig. 7a) (Jo *et al*., 2023). These strong interactions will result in a stable ternary complex preventing reannealing of DNA within the cell.

Recent studies have determined due to exatecan possessing an amine benzyl ring two additional hydrogen bonds will be formed between the amine group of exatecan and the topoisomerase I-DNA

complex in comparison to those made with camptothecin. The oxygen and nitrogen groups bearing hydrogen within exatecan will form hydrogen bonds with amino acid residues Asn722, Arg364, Asp533 and Asn352 present within topoisomerase I (Jo *et al*., 2023) (fig. 7b). Hydrogen bonds are also formed between the base oxygen on DNA and hydrogen present on the benzyl amine group of exatecan (fig. 7b) (Jo *et al.,* 2023). Aromatic rings found in both DNA and exatecan also allow for π- π stacking of adjacent base pairs, these strong interactions enhance the binding affinity of exatecan.

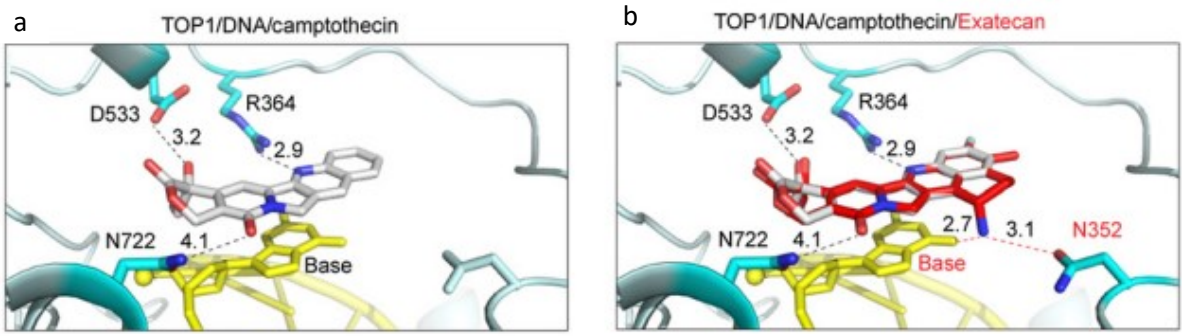


Figure 7 – View of compounds bound to topoisomerase I (cyan) and DNA (yellow) including distances of the bonds (a) Camptothecin (grey) (b) Exatecan (red) superimposed on camptothecin. Adapted from Jo *et al*. (2023). Used with permission of American Association for Cancer Research, conveyed through Copyright Clearance Center, Inc.

DXd has a similar chemical structure to exatecan (fig. 3) replacing the amine group present within exatecan with an amide and alcohol group. The similarities in structure will allow for DXd to form similar interactions with the topoisomerase I-DNA complex to those exatecan displays, identified by Jo *et al*. (2023). These similar interactions would lead to DXd having a similar efficacy to exatecan which is supported by IC_{50} values (half-maximal inhibitory concentration) recorded by Ogitani *et al*. (2016), DXd was found to have a IC_{50} value of 0.31 µmol/L and exatecan 0.25 µmol/L.

3.2 – Antibody-dependent cell-mediated cytotoxicity

Although the initial MOA of T-DXd is to undergo endocytosis into the tumour cells the antibody trastuzumab also stimulates a further MOA improving its efficacy. Natural killer (NK) cells found within the body can complete antibody-dependant cell-mediated cytotoxicity, a cell-mediated immune defence mechanism. Fc receptors on the NK cells will bind via intermolecular bonds to the Fc region (fig. 8) of trastuzumab whilst bound to the HER2 antigens on the tumour cells surface (Guti *et al*., 2022). Binding triggers the release of proteolytic enzymes leading to cell apoptosis through the destruction of the cell's cytoskeleton proteins and chromosomal degradation (Guti *et al*., 2022). Topoisomerase inhibitors have also been found to increase the sensitivity of tumour cells to T-cell-mediated cytotoxicity increasing their antitumour efficacy (Mckenzie *et al*., 2018).

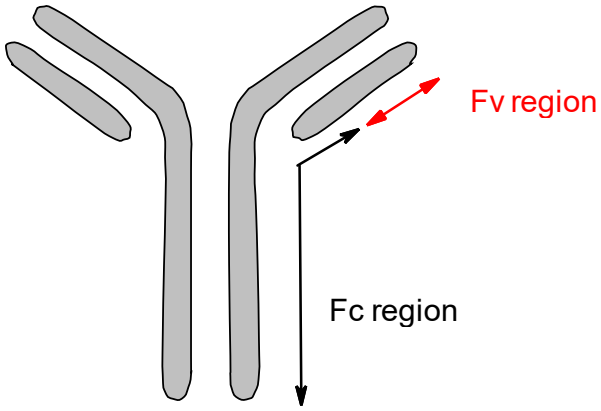


Figure 8 – An image to show the Fc region of a mAb.

3.3 – Bystander effect

A drugs membrane permeability often correlates to its *in vivo* efficacy, as a result if a drug is highly membrane permeable it will see improved *in vivo* efficacy in comparison to a drug with low membrane permeability (Bennion *et al.,* 2017). The membrane permeability of compounds has been found to increase with increasing hydrophobicity and although this leads to a potent bystander effect issues may arise as discussed earlier with potential toxic side effects (Buecheler *et al.,* 2018) if the correct linker is not used.

The cytotoxic payload released from T-DXd, DXd, after endocytosis and enzyme cleavage is highly potent and membrane permeable therefore has been found to exert a bystander effect. As DXd is no longer bound to trastuzumab in its ADC form it is no longer required to bind to the HER2 antigens present on the cell. DXd has shown outstanding membrane permeability as a result once released within the cell DXd may diffuse into neighbouring cells whether they are HER2 positive or HER2 negative tumour cells (Suzuki *et al*., 2021). This potent MOA has improved the drug's efficacy results throughout clinical trials.

3.4 – Blockage of dimerization

Scientific research groups whose study is solely based on T-DXd do not discuss blockage of dimerization, they focus on endocytosis, antibody-dependant cell-mediated cytotoxicity and a potent bystander effect induced by DXd. However scientific research papers which have investigated trastuzumab individually have identified its ability to block dimerization (Maadi and Wang, 2022).

HER2 are known as tumour associated antigens, lacking ligands therefore overexpression increases receptor density causing activation via homodimerization and transduction of growth-promoting signals (Sheikh and Huang, 2023). HER2 positive breast cancer cells can produce a variation of HER receptor dimers however as trastuzumab only binds to HER2, epidermal growth factor receptor (EGFR)-HER2 heterodimers and HER2-HER2 homodimers are the focus of recent studies (Zhao *et al*., 2021; Maadi and Wang, 2022). HER2 is part of the tyrosine kinase receptor family and can function both as a receiver or an activator (Maadi and Wang, 2022).

Research on the HER2-HER2 homodimer completed by Zhao *et al*. (2021) discovered that the binding of trastuzumab to HER2 exerted both agonistic and antagonistic effects dependant on the function of HER2. When HER2 is functioning as a receiver trastuzumab was found to inhibit dimerization between HER2 and other HER receptors therefore acting as an antagonist (Zhao *et al*., 2021). On the

other hand, trastuzumab will act as an agonist when binding to HER2 functioning as an activator inducing structural changes to the tyrosine kinase domain.

Maadi and Wang (2022) completed studies around the EGFR-HER2 heterodimers formed due to epidermal growth factor (EGF) stimulation. Their studies showed that trastuzumab will inhibit EGF-induced lipid raft localization of the heterodimer subsequently inhibiting protein kinase B phosphorylation and cell proliferation rather than block the dimerization of the heterodimer. Trastuzumab was found to block cell proliferation in the G1 phase when the cell is preparing to divide (Maadi and Wang, 2022).

3.5 - Recent advances

As discussed previously Zhang *et al*. (2021) completed research into the utilization of the functional groups present on trastuzumab allowing conjugation of two different cytotoxic payloads. Nilchan *et al.* (2019) also researched the production of a dual-drug ADC in efforts to mimic the combined usage of drugs during cancer chemotherapies to reduce drug resistance and improve the outcomes of the patient's treatment. Researchers paired two drugs with different MOA, monomethyl auristatin F (MMAF) and PNU-159682, conjugating them to a suitable mAb (Nilchan *et al*., 2019). They discovered that although the combination of both drugs did not work synergistically both drugs successfully exhibited individual MOA towards the targeted HER2 tumour cells.

Similar studies were completed by Kumar *et al.* (2018) conjugating MMAE and pyrrolobenzodiazepine dimer (PBD) to the antibody trastuzumab. Research completed to investigate the dual mechanistic action of the two drugs produced the same results as those determined by Nilchan *et al.* (2019). Although research completed by both groups produced the same results their conjugation methods varied. Nilchan *et al*. (2019) used two linkers conjugating the cytotoxic payloads to different sites on the mAb (fig. 9a). On the other hand, Kumar *et al.* (2018) produced a single linker conjugating both cytotoxic payloads to the same linker (fig. 9b).

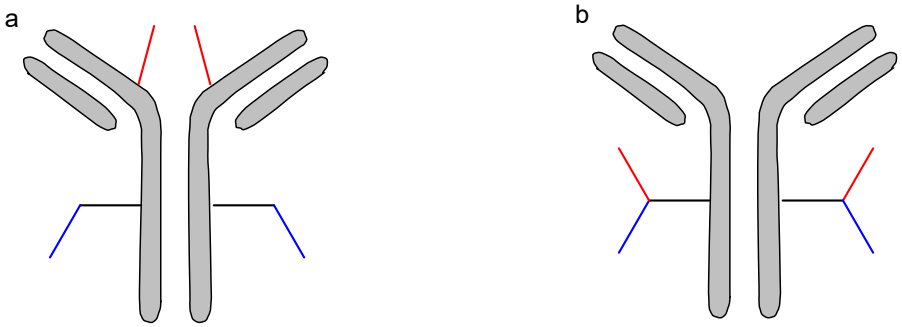


Figure 9 - The structure of two dual-mechanistic ADCs (a) Adapted from Nilchan *et al*. (2019) (b) Adapted from Kumar *et al.* (2018)

Research has also investigated the conjugation of an immune checkpoint antibody and a noncytotoxic immune modulator to produce immune modulating ADCs (IM-ADC) (He *et al*., 2021). These ADCs can trigger an antitumor immune response activating specific receptors, reshape immunosuppressive microenvironments and increase cell sensitivity (He *et al*., 2021). He *et al.* (2021) also discovered that due to the vast number of MOAs the IM-ADC can undergo it has potential to treat chronic viral infections including HIV or HBV providing an alternate usage for ADCs.

3.6 – Where next

Although many scientific papers state the same MOA for T-DXd there is still uncertainty to if the true MOA of T-DXd has been discovered therefore future research should be carried out to clarify this. Gaining clarity on T-DXds' true MOA will allow for correct usage of the drug and future development of ADCs with a similar successful MOA. Due to the lack of clarity surrounding the MOA of T-DXd, research outlining the interactions between DXd, and the topoisomerase I-DNA complex is minimal. Further research around these interactions will lead to clarity on how the structure of DXd leads to such potent cell apoptosis. These discoveries will allow for development into further structural improvements of cytotoxic payloads which target topoisomerase I.

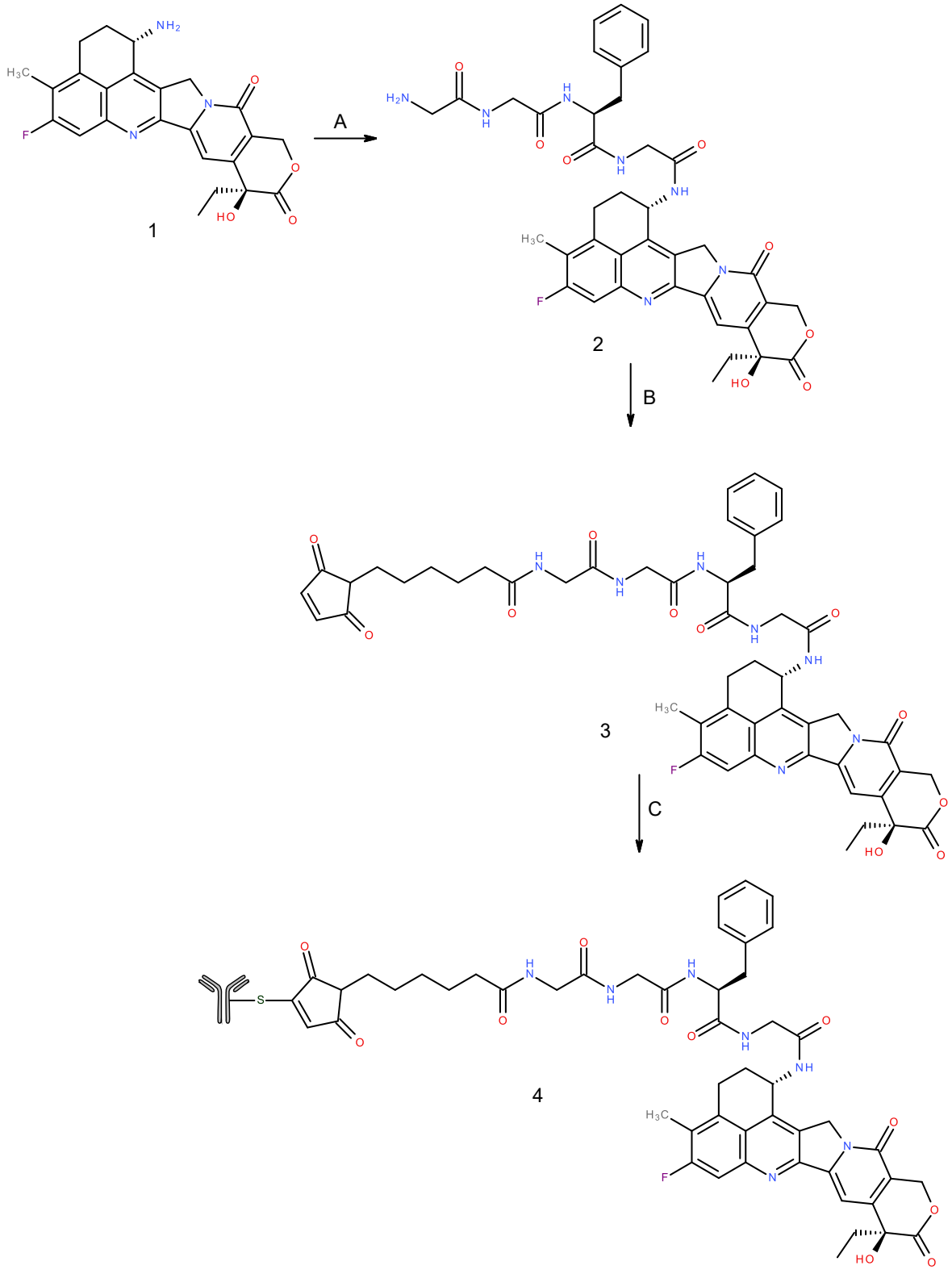
Research into the dual conjugation of cytotoxic payloads to ADCs has shown positive advancements demonstrating the extent to which ADCs can be utilized in the delivery of previously classified toxic drugs. Future research should determine if alterations to the dual conjugated ADCs structure such as changes in the linker used or alternative drug pairing can increase the ADCs efficacy by allowing synergistic work of the conjugated drugs in comparison to each drug completing their own MOA.

Chapter 4

Synthesis

4.1 – Existing synthetic routes

ADC synthesis uses conjugation chemistry to bind cytotoxic drugs to a mAb as a result their synthesis is complex and takes place through multiple steps. Nakada *et al*. (2016) reported a synthetic approach to producing trastuzumab ADCs bearing exatecan derivative payloads however this synthesis begun from exatecan as a pre bought product.

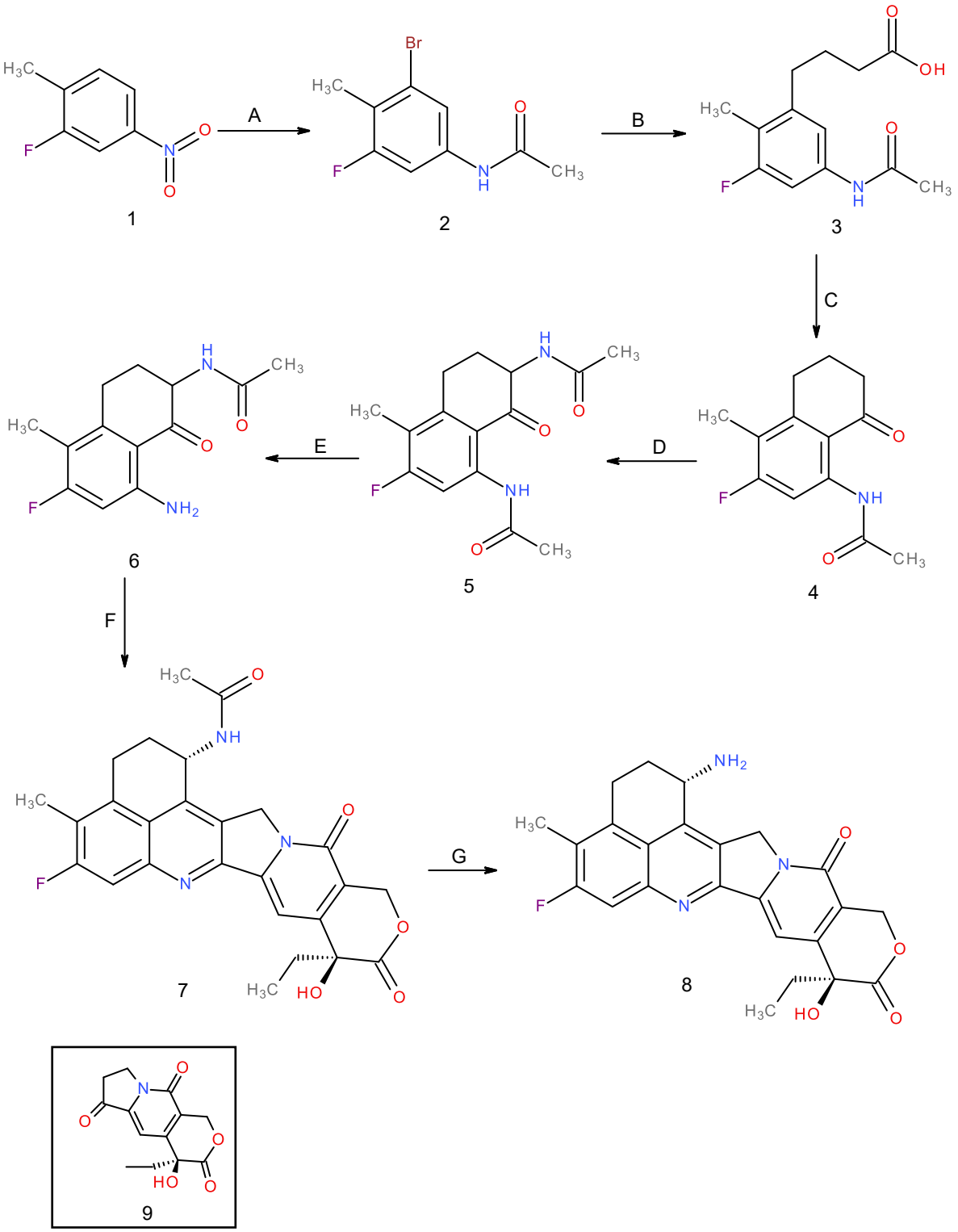


Scheme 1 – The synthesis of trastuzumab ADCs containing exatecan. Adapted from Nakada *et al*. (2016) Used with permission of Elsevier Science & Technology Journals conveyed through Copyright Clearance Center, Inc. **(A) 1.** Boc-GGFG-OH, WSCl, Et_3N/DMF **2.** TFA/CH_2Cl_2; **(B)** mc-Osu, Et_3N/DMF; **(C)** TCEP HCl.

Key steps within synthesis:

- C-C bond forming between exatecan and Boc-GGFG-OH (step A);
- Deprotection of Boc-GGFG-exatecan and condensation with 6-maleimidohexanoic acid N-hydroxysuccinimide ester (mc-Osu) (step B); and
- Conjugation by reduction of the disulfide groups on the mAb using TCEP HCl (step C).

However, in more recent studies Yoshio *et al*. (2020) identified areas for improvement within the existing synthesis such as repetition of oxidation and reduction reactions and ring-opening and ring-closing reactions leading to the long complex synthesis. Yoshio *et al*. (2020) have reported an improved synthetic route for T-DXd which is more suitable for industrial production, by implementing late stage coupling of exatecan to the linker.



Scheme 2 – Synthesis of exatecan. Adapted from Yoshio *et al*. (2020). **(A) 1.** NBS, H_2SO_4, heptane, 60°C **2**. H_2, Pt/C, EtOAc, rt **3.** TEA, Ac_2O, EtOAc, rt, 37% yield for step A; **(B) 1**. 3-butenoic acid, $Pd(OAc)_2$, P(*o*-tol)$_3$, DIPEA, THF, H_2O **2.** H_2, Pd/C, 2-MeTHF, 40°C, 91% yield for step B; **(C)** TFA, TFAA, 4°C, 92% yield; **(D) 1.** amyl nitrite, *t*-BuOK, THF, 4 **2.** H_2, Pt/C, Ac_2O, HOAc, rt, 52% yield; **(E)** 2 N HCl, EtOH, rt to 50°C, 80% yield; **(F)** 9, PPTS, *o*-cresol, $PhCH_3$, 96% yield; **(G)** MsOH, ethylcyclohexene, 2-methoxyethanol, H_2O 43% yield.

Key steps within the synthesis:

- Bromination, nitro reduction and acetylation of compound 1 to give compound 2 (step A);
- C-C bond formation (step B);
- Activation of the carboxylic acid on compound 3 for intramolecular cyclization (step C);
- Nitration and heterogenous reduction of compound 4 (step D);
- Aniline deacetylation of compound 5 (step E);
- PPTS-mediated Friedlaender-type reaction and dehydrative intramolecular cyclization with compound 9 (step F); and
- Reflux of mixture producing two isomers (step G).

Yoshio *et al*. (2020) determined during their studies that the synthesis of exatecan (8) in step G produced two diastereoisomers (fig. 10), the desirable exatecan isomer and the undesirable *epi*-exatecan isomer. The formation of the two isomers as can be seen from figure 10 is determined by the configuration of the amine group attached to the cyclic ring which may be in front or behind the plane. Although both isomers are produced during the synthesis and can be isolated from one another it was determined that the undesirable *epi*-isomer can also undergo amine reprotection and deprotection sequences to be converted to the desirable configuration (Yoshio *et al*., 2020).

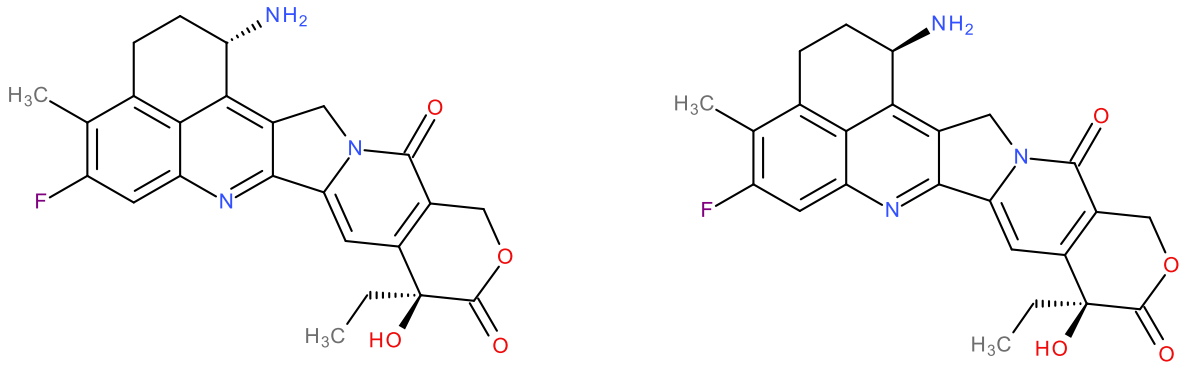
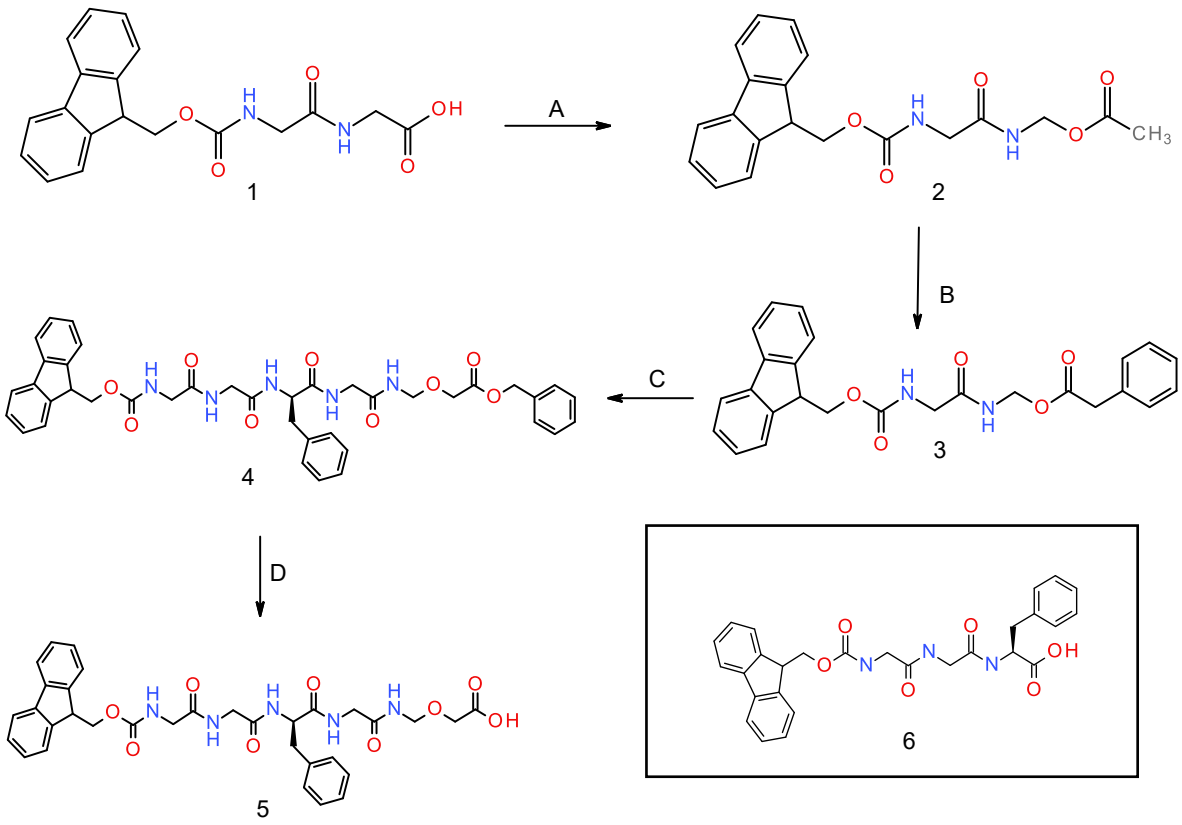


Figure 10 – The structure of two exatecan isomers produced during exatecan synthesis. Adapted from Yoshio *et al*. (2020).

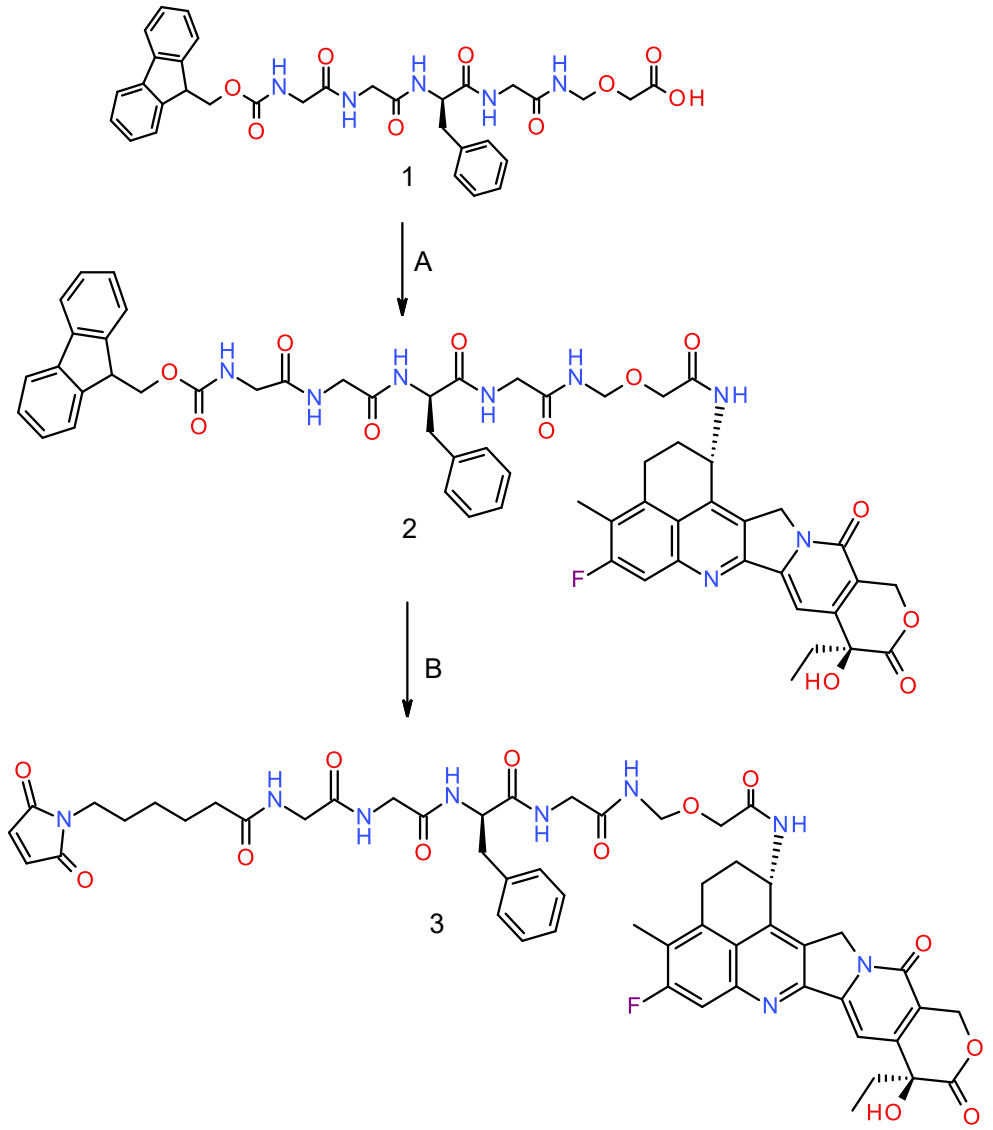
Studies by Li *et al*. (2019) used 3-fluoro-4-methylaniline as a starting material to synthesize exatecan derivative ADCs, this starting material is very similar to that used during Yoshio *et al*. (2020) studies (scheme 2). However, the aim of Li *et al*. (2019) studies was to produce an ADC bearing an exatecan derivative without the amine bearing cyclic ring therefore determining if the ring had any influence on the efficacy of the ADCs. Due to the removal of the amine bearing ring, which presented a chiral centre within the compound, Li *et al*. (2019) did not report the production of exatecan isomers throughout the synthesis of the cytotoxic payloads and determined the ring had no significant influence on efficacy.



Scheme 3 – The synthesis of the deruxtecan linker intermediate. Adapted from Yoshio *et al*. (2020). **(A)** $Pb(OAc)_4$, HOAc, THF, 68% yield; **(B)** benzyl glycolate, $B(C_6F_5)_3$, 1,2-DME, 0°C, 88% yield; **(C) 1.** DBU, DMA, rt **2.** 6, EDCl, HOBt·H_2O, PPTS, rt, 94% yield; **(D)** H_2, Palladium carbon-ethylenediamine complex, THF, H_2O, rt, 89% yield.

Key steps within synthesis:

- Oxidative decarboxylation under reflux conditions (step A);
- Acetyl cleavage and two-carbon homologation under Lewis acid conditions (step B);
- Fmoc removal and coupling to 6 (step C); and
- Cleavage of benzyl ester (step D).



Scheme 4 – The synthesis of the deruxtecan. Adapted from Yoshio *et al*. (2020). **(A)** Exatecan, EDCl, HOBt·H_2O, TEA, DMSO, THF, rt, 84% yield; **(B) 1.** DBU, THF **2.** mc-Osu, PPTS, TEA, pyr., CH_3CN, THF.

Key steps within synthesis:

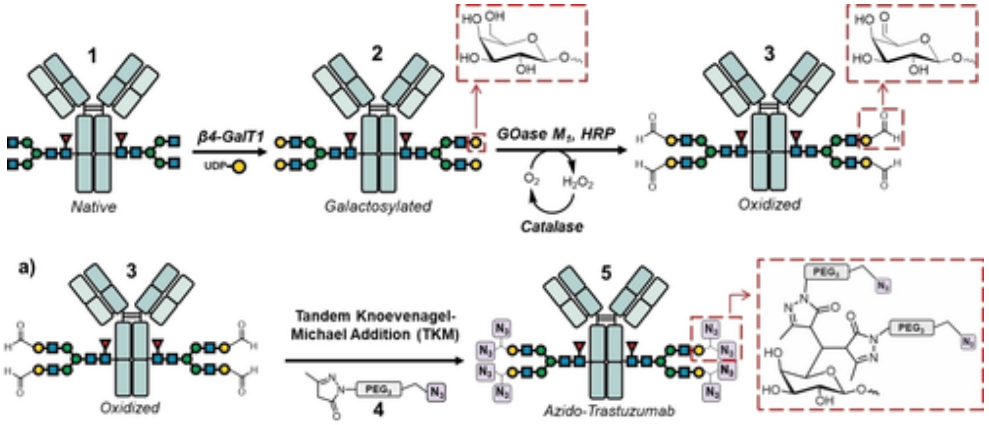
- Peptide coupling of exatecan and deruxtecan linker intermediate (step A); and
- Fmoc deprotection of compound 2 and condensation with mc-Osu (step B).

Conjugation to the mAb trastuzumab was completed under the same conditions outlined by Nakada *et al*. (2016), cysteine bioconjugation by reduction of the disulfide groups using TCEP HCl. Cysteine conjugation allows for high specificity in comparison to lysine bioconjugation methods due to the limited number of reactive cysteine residues present on the mAb (Bryden *et al*., 2018) and the high nucleophilicity of deprotonated thiolate side chains (Walsh *et al*., 2021).

Although both schemes take different routes of synthesising trastuzumab ADCs there are some similarities in the reagents and reactions taking place. Both studies used C-C bond formation to extend the exatecan molecule and deprotection followed by condensation with mc-Osu to produce the exatecan-linker molecule.

4.2 – Recent advances in antibody-drug conjugates synthesis

Recent approval of T-DXd has shown the clinical benefits of ADCs with high DARs leading to further studies aiming to improve and simplify the synthesis of high DAR ADCs. Most ADC conjugation methods use cysteine modification (Nakada *et al*., 2016; Yoshio *et al*., 2020) however this limits the conjugation to one drug per cysteine residue present on the mAb (Kumar *et al*., 2020). A DAR value of eight is the theoretical maximum DAR for ADCs formed through conventional interchain cysteine conjugation (Buecheler et al., 2018). Angelasto *et al*. (2022) recently investigated alternative methods of conjugation to trastuzumab using methods of biocatalysis followed by tandem Knoevenagel-Micheal addition (TKM) chemistry with the aim to improve the DAR of ADCs. The *N*-glycan chains of the mAb trastuzumab were the targeted conjugation site of the linker payloads throughout the synthesis. Angelastro *et al*. (2022) utilized the reactivity of aldehyde groups to conjugate the cytotoxic drugs to each N-glycan chain present on trastuzumab.



Scheme 5 – The synthesis of ADCs bearing the mAb trastuzumab by galactose oxidase. (Angelastro *et al*., 2022).

Key steps within the synthesis:

- Transfer of D-galactose units from UDP-Gal to each N-glycan chain on trastuzumab (step A);
- Enzymatic oxidation of C6-hydroxy group of D-galactose into C6-aldehyde by galactose oxidase (step B); and
- Ligation to each aldehyde group of two linker-payload via TKM (step C).

These methods of conjugation gave an average DAR value of 7.3 showing the synthetic route is successful in producing ADCs with high DARs (Angelasto *et al*., 2022).

4.3 – Where next

The syntheses outlined above are all multi-step showing the difficulties faced during the manufacture of T-DXd and ADCs in general due to their complex structure. Although Yoshio *et al*. (2020) has outlined a reduced synthetic route further improvements in the synthesis should be researched to reduce this further to improve the production of T-DXd on a larger scale.

As discussed previously high DAR ADCs have shown improved clinical results in comparison to low DAR ADCs. Future research should therefore investigate alternative methods of conjugation enhancing the DAR of ADCs and incorporating masking linkers.

Chapter 5

Conclusions and recommendations

5.1 – Conclusions

The predominant impact cancer has on millions of people globally demonstrates the urgent need to research efficient methods of drug delivery to overcome off target specificity of drugs found to successfully treat cancer patients. ADCs have already shown promising clinical results to overcome these limitations however further develops are required to increase their usage and prevent drug resistance. Dual conjugated ADCs have shown promising outcomes to develop ADCs potency further by allowing the delivery of two drugs with different MOAs. Furthermore, efforts to mask ADCs hydrophobicity have shown improvements in DARs and efficacy increasing metabolic stability and water solubility.

The objectives of this paper were to review the structure, MOA and synthesis of T-DXd and identify suitable suggestions for future research. The structure of T-DXd and its MOA have been reviewed and recent advances to improve ADCs structure and MOA have been discussed. Recent developments in the synthesis of T-DXd have been reviewed and set out. Future recommendations for research into ADCs structure, MOA and synthesis have been suggested throughout and summarised below.

5.2 – Future recommendations

This paper has reviewed one of thirteen ADCs currently approved by the FDA to treat cancer patients and has suggested potential future research areas into its structure and reviewed improvements in its synthesis. It is recommended that further research into drug linkers capable of masking the hydrophobicity of high DAR ADCs is completed.

Although the scientific papers reviewed identify similar MOAs of T-DXd many state that its MOA is still unclear. It is recommended that further research is completed to clarify the MOA of T-DXd and to identify the interactions between DXd and the topoisomerase I-DNA complex to allow for future efficacy improvements and drug development.

Relative to the synthesis of T-DXd and ADCs, this paper has identified the key synthetic steps and highlighted the most recent research to improve the synthesis of ADCs specifically with high DAR values. It is recommended that research continues to look at improvements of these synthetic steps due to the clinical benefits ADCs with high DARs can provide. These methods of synthesis should also aim to incorporate masking drug linkers to reduce the ADCs hydrophobicity improving the ADCs metabolic stability and reducing potential for off target specificity.

Although significant improvements have been made over the past decades cancer still affects millions around the world therefore development into its treatment should be vital. ADCs show promising results as a treatment method however the potential areas for research signposted throughout this paper should be completed to ensure their continued development to treat cancer more efficiently.

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