Review Article

Novel development strategies and challenges for anti-Her2 antibody-drug conjugates

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ABSTRACT

Antibody-drug conjugates (ADCs) combining potent cytotoxicity of small-molecule drugs with the selectivity and excellent pharmacokinetic profile of monoclonal antibody (mAb) are promising therapeutic modalities for a diverse range of cancers. Owing to overexpression in a wide range of tumors, human epidermal growth factor receptor 2 (Her2) is one of the most utilized targeting antigens for ADCs to treat Her2-positive cancers. Owing to the high density of Her2 antigens on the tumor cells and high affinity and high internalization capacity of corresponding antibodies, 56 anti-Her2 ADCs which applied >10 different types of novel payloads had entered preclinical or clinical trials. Seven of 12 Food and Drug Administration (FDA)-approved ADCs including Polivy (2019), Padcev (2019), EnHertu (2019), Trodelvy (2020), Blenrep (2020), Zynlonta (2021), and Tivdak) (2021) have been approved by FDA in the past three years alone, indicating that the maturing of ADC technology brings more productive clinical outcomes. This review, focusing on the anti-Her2 ADCs in clinical trials or on the market, discusses the strategies to select antibody formats, the linkages between linker and mAb, and effective payloads with particular release and action mechanisms for a good clinical outcome.

Statement of Significance: The in-depth analysis of typical anti-Her2 ADCs reveals some key characteristics of successful anti-Her2 ADCs, including antibody formats, linkage strategies between linker and mAbs, and selection of innovative payloads.

INTRODUCTION

Human epidermal growth factor receptor-2 (Her2), a transmembrane receptor of tyrosine kinases, is one member of the epidermal growth factor receptor (EGFR) family which also includes Her1 (also known as EGFR), Her3, and Her4 [1]. Each of them comprises an extracellular domain, a transmembrane lipophilic helix, and an intracellular tyrosine kinase domain (except for Her3) [2]. All four members are essential for regulating cell proliferation and differentiation through active formats upon ligand-dependent or independent homo or hetero-dimerization [3] (Fig. 1). However, unlike the other three members, the extracellular domain of Her2 is the only one to dimerize with any of the other members in the activated state without binding ligand and then trigger signaling pathways that regulate cell proliferation and survival [4]. Meanwhile, accumulating evidence indicates that Her2 is overexpressed in a variety of cancers, such as breast cancer [5], gastric cancer [6], lung cancer [7], and ovarian cancer [8]. Especially, up to 15% of breast cancer patients have Her2
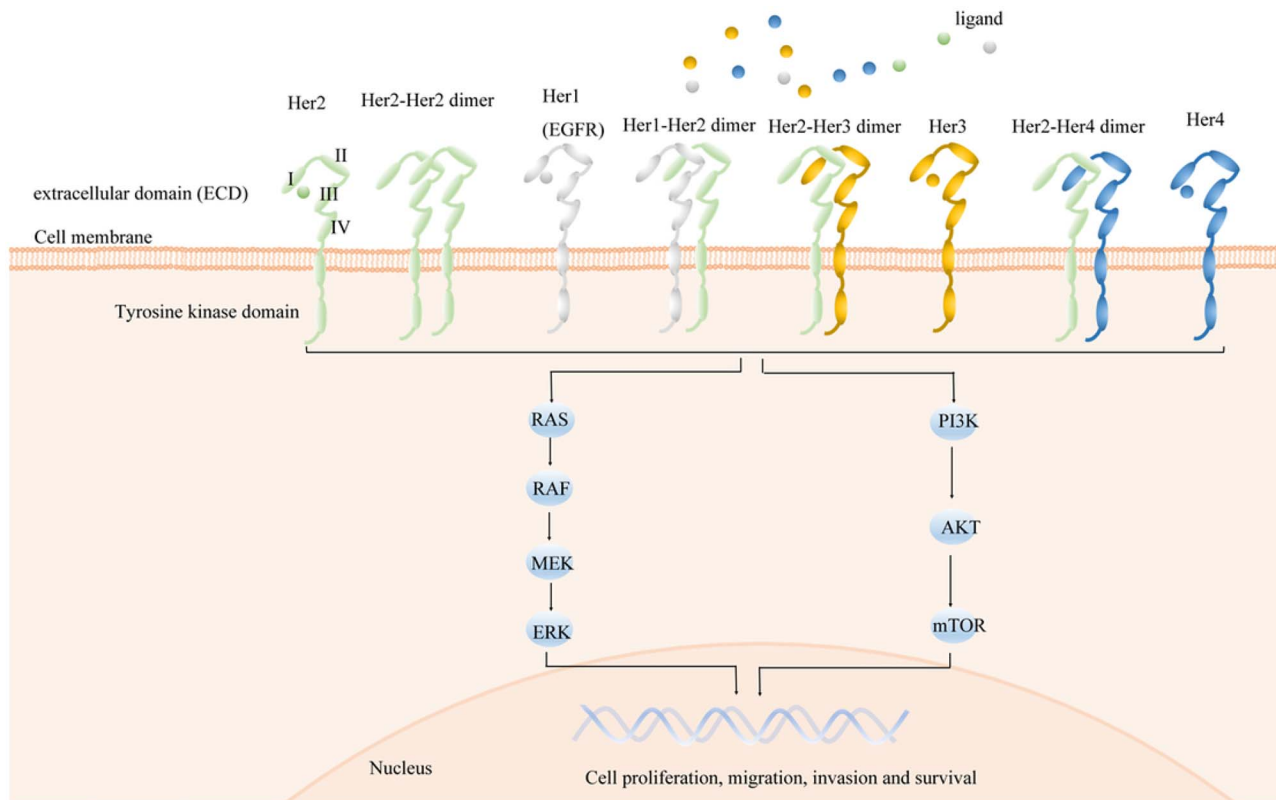
Figure 1. Schematic representation of Her2 signaling pathway.

gene amplification and overexpression [9]. Therefore, Her2 has been regarded as a valuable prognostic factor for breast cancer as well as a potent therapeutic target for treating various Her2-positive cancers. Thirty anti-Her2 antibody-drug conjugate (ADC) candidates have entered clinical testing. To date, 23 among them are still making good progress, and three of them have been approved for marketing (Table 1). In addition, seven ADCs that have been declared terminated are listed in Table 2. The above data show that the failure rate of anti-Her2 ADCs is not high (23.3%), indicating that Her2 is a highly desirable target of ADCs. In this review, we discussed the prevalent approaches for selection of engineering monoclonal antibody (mAb), linkage techniques, payloads, drug release-mechanism basing on anti-Her2 ADCs in clinical trials or on the market.

SELECTION OF ANTIBODY FORMATS

Homogeneity and compatibility of the antibody are stringent for improving ADCs to reduce the attrition of drug candidates [10]. Currently, >550 immunoglobulins (IgGs) and their derivatives have been assessed in clinical studies for various indications [11], and then most approved antibodies used in the ADCs are mainly adopted from three IgG isotypes (IgG1, IgG2, and IgG4) due to their high avidity for the target antigen and their long circulatory half-life in the blood [12–14]. Besides the difference of heavy chain amino acid sequences, the three IgG isotypes are also different in interchain disulfides and effector functions

including Antibody-Dependent Cellular Cytotoxicity (ADCC), Complement-Dependent Cytotoxicity (CDC), and Antibody-Dependent Cellular Phagocytosis, which provide different strategies for generating ADCs.

IgG1 isotype usually presents more power on ADCC and CDC functions than IgG2 and IgG4 isotypes [15], IgG2 isotype commonly showcases the accessibility to conjugate more payloads because of more interchain disulfides than IgG1 and IgG4 isotypes [16, 17], and IgG4 isotypes can form half molecules (one heavy chain and one light chain) and exchange Fab-arm with different specific antibodies to produce bispecific antibodies both *in vitro* and *in vivo* [18]. To date, IgG1 isotype and its derivatives are widely utilized in the majority (>20) of anti-Her2 ADCs including trastuzumab emtansine [19] and trastuzumab deruxtecan [20].

The bispecific antibody (bsAb), designed to recognize and bind two different epitopes or antigens and to realize therapeutic functions up to now unattainable by traditional antibody formats [21], has provided novel promising strategies for the development of ADCs, as well as anti-Her2 ADCs (such as ZW49 and MEDI4267) [22]. The molecular architecture of bsAb formats can be commonly categorized into fragment-based formats and Fc-based formats, which consist of two or more antibody fragments (usually scFv, Fab, or single-domain Ab) and compose of homo- or heterodimeric Fc domains, respectively [21]. For example, the biparatopic antibody used in the monospecific, tetravalent ADC MEDI4267, is generated by combining the single-chain variable fragment (scFv) of trastuzumab with the N

Table 1. List of anti-Her2 ADCs in clinical trials or on the market*

Name	Antibody	Payload	DAR	Linkage	Indication	ClinicalTrials.ID	Phase
Trastuzumab emtansine	Trastuzumab (IgG1)	DM1 (Maytansine)	3.5	Lysine-SMCC	Metastatic breast cancer	-	Approved
Trastuzumab deruxtecan	Trastuzumab (IgG1)	DXd (Topoisomerase I inhibitor)	8.0	Cysteine-maleimide	Metastatic breast cancer	-	Approved
Disitamab vedotin(RC48)	Disitamab (IgG1)	MMAE (Auristatin)	4.0	Cysteine-maleimide	Metastatic urothelial cancer	-	Approved in China
Trastuzumab duocarmazine	Trastuzumab (IgG1)	DUBA (Duocarmycin)	2.8	Cysteine-maleimide	Metastatic breast cancer	NCT03262935	3
TAA013	Trastuzumab (IgG1)	DM1 (Maytansine)	3.5	Lysine-SMCC	Metastatic breast cancer	CTR20200806	3
ZRC-3256	Trastuzumab (IgG1)	DM1 (Maytansine)	-	Lysine-SMCC	Metastatic breast cancer	CTRI/2018/07/014881	3
MRG002	IgG1	MMAE (Auristatin)	-	Unknown	Advanced solid tumors	NCT04492488	2
ARX788	Transtuzumab (IgG1)	MMAF(Auristatin)	1.9	pAF-hydroxylamine-PEG4	Metastatic breast cancer/gastric cancer	CTR20201708	2/3
BDC-1001	Trastuzumab (IgG1)	TLR7/8 agonist	-	Unknown	Metastatic breast cancer/gastric cancer	NCT04278144	1/2
A166	Trastuzumab (IgG1)	MMAF (Auristatin)	2	Lysine site-specific	Metastatic breast cancer	NCT03602079	1/2
FS-1502	Trastuzumab (IgG1)	MMAF (Auristatin)	2.0	Cysteine-maleimide	Breast cancer	NCT03944499	1
SHR-A1201	Trastuzumab (IgG1)	DM1 (Maytansine)	-	Lysine SMCC	Metastatic breast cancer	CTR20191558	1
DP303c	Trastuzumab (IgG1)	DP104n	2.0	Unknown	Gastric cancer	NCT04146610	2
BI-CON-02	Trastuzumab (IgG1)	Unknown	-	Unknown	Metastatic breast cancer	NCT03062007	1
ALT-P7	Trastuzumab biobetter HM2 (IgG1)	MMAE (Auristatin)	2.0	Cysteine-maleimide	Metastatic breast cancer	NCT03281824	1
DX126–262	DX-CHO9 (IgG1)	Tubulysin	-	Cysteine-maleimide	Breast/gastric cancer	CTR20191224	1
ZW49	IgG1	Auristatin	-	Cysteine-MC	Metastatic cancers	NCT03821233	1
HS630	Trastuzumab (IgG1)	DM1 (Maytansine)	-	Unknown	Breast cancer	CTR20181755	1
B003	Trastuzumab (IgG1)	DM1 (Maytansine)	-	Lysine-SMCC	Metastatic breast cancer	NCT03953833	1
SBT6050	IgG1	TLR8 agonist	-	Unknown	Advanced solid tumors	NCT04460456	1
SHR-A1811	Unknown	Unknown	-	Unknown	Advanced solid tumors	NCT04446260	1/2
MT-5111	Unknown	SLTA	-	Unknown	Advanced solid tumors	NCT04029922	1
GQ1001	Unknown	DM1 (Maytansine)	2.0	Unknown	Advanced solid tumors	NCT04450732	1

*Updated on 01 2021; Her2: Human epidermal growth factor 2; pAF: para-acetylphenylalanine; SMCC: succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate; SLTA: Shiga-like toxin A; Most Data from Beacon-targeted therapies: https://www.beacon-intelligence.com/

Table 2. List of anti-Her2 ADCs failed in clinic settings*

Name	Antibody	Payload	DAR	Linkage	Indication	Phase
XMT-1522	HT-19	Auristatin F-HPA (Auristatin)	12	Cysteine-fleximer polymer	Breast/gallbladder/gastric cancer	1
ADCT-502	Trastuzumab	SG3199 (PBD)	1.7	Cysteine site-specific	Breast/bladder/gastric cancer	1
MEDI4276 (Bispecific)	Trastuzumab& 39S	AZ13599185 (Tubulysin)	4	Cysteine site-specific	Breast/gastric cancer	1
NJH395	Unknown	TLR7 agonist	Unknown	Unknown	Solid tumors	1
MM-302	F5	Doxorubicin	Unknown	Unknown	Advanced solid tumors	2/3
BAT8001	BAT0606	Maytansinoid	3.5	Cysteine-3AA	Advanced solid tumors	3
DHES0815A	Unknown	PBD	Unknown	Unknown	Breast cancer	1

*Updated on 22 Oct 2021.

terminus of the heavy chain of the anti-Her2 fully human mAb 39S (IgG1κ), which provides four antigen-binding sites to induce potent receptor clustering and internalization [23].

Currently, nanobodies (Nbs), characterized by high affinities, robust structures, high hydrophilicity, low off-target aggregation, and deep tissue penetration, have attracted enormous attention in the fields of biopharmacy [24]. Furthermore, great operability in genetically or chemically engineered and easy availability through large synthetic/naïve or immunized cDNA-libraries of Nbs facilitates the commercializing of Nbs for diagnostic and therapeutic application [25, 26]. Wu *et al.* designed a Nb-based drug delivery system, which tethered site-specific PEGylation of anti-EGFR Nbs to human serum albumin coated upconversion nanoparticles and loaded anticancer drug doxorubicin, and the results demonstrated superior specificity and high anti-proliferation effect to the EGFR-positive tumor cells *in vitro* [27].

NOVEL PAYLOADS USED IN ANTI-HER-2 ADCS

So far, the most common payloads of ADCs are cytotoxins and several criteria have to be met for qualified cytotoxins

to gain the ideal tumor suppressing effects: (a) high potency limited number of antigens on the target cell surface, and limited endocytosis efficiency of ADCs result in limited cytotoxins transporting into the cell, hence cytotoxins must possess enough potency to kill tumor cells; (b) enough capacity of hydrophilicity most cytotoxins are hydrophobic, hence we need to improve their hydrophilic ability when modified to connect with linkers [28] to avoid the tendency of ADC aggregation and so on. Over a long period, only a few types of cytotoxins had been adopted as the payloads of ADCs [29]. With the increasingly fierce competition in this field, >10 new cytotoxins are used as the payloads of anti-Her2 ADCs which are being evaluated in the preclinical or early clinical trials, and the detailed features of some of the ADCs have not yet been published (Table 2).

Payloads targeting microtubules

Tubulin inhibitors, especially auristatins and maytansine, were adopted as the payloads of most ADCs in clinical trials. These cytotoxins target and inhibit dynamic microtubules during the spindle formation and then further induce apoptosis in mitotic cells [30, 31]. The typical representatives of auristatins are monomethyl auristatin E (MMAE) which shows significant plasticity for optimizing their physical and chemical properties and retain their potency when conjugated with mAbs due to their unique linear pentapeptides and non-natural amino acids [30]. The typical representatives of maytansine are DM1. From the statistical results in Table 1, it is not difficult to find that MMAE and DM1 were the frequently used types of payloads of anti-Her2 ADCs in the clinical trials. In addition, 5 of 12 approved ADCs (Adcetris, Kadcyla, Polivy, Padcev, and Tivdak) adopted MMAE or DM1 [11, 32–34]. Notably, PF-06804103 was the only ADC that used Monomethyl auristatin D (MMAD) as the payload but was discontinued in phase I. MMAD is a new auristatin derivative with a similar structure to MMAE. The difference between MMAD and MMAE is that the C-terminal of MMAD has a dolaphenine residue which is susceptible to cleavage in rodent plasma [35].

Payloads targeting DNA

The Deoxyribonucleic acid (DNA)-targeting agents, such as calicheamicins, duocarmycins, and pyrrolobenzodiazepines (PBDs), can irreversibly attach to the minor groove of DNA through covalent bonds, thereby destroying the dimensional structures of DNA and further leading to cell death. Different from tubulin inhibitors which only kill mitotic phase tumor cells, DNA-targeting agents could exert on proliferating and non-proliferating cells [36]. Duocarmazine, a derivative of duocarmycins, was conjugated to trastuzumab to generate an ADC named SYD985 and its antitumor activity in breast cancers had been elucidated in clinical trials [37]. PBD dimers with exceptionally potent activity against many tumor cells triggered enthusiasm in the development of ADCs. However, the clinical trials to evaluate the pharmacokinetics, safety, and tolerability of ADCT-502 and DHES0815A, which conjugated with PBD, in patients with Her2-positive breast cancer had been

terminated due to poor efficacy and/or safety concerns [38, 39]. Calicheamicins, a potent antitumor antibiotic, had been used in two approved ADCs, gemtuzumab ozogamicin (an anti-CD33 ADC) and inotuzumab ozogamicin (an anti-CD22 ADC). However, the use of calicheamicins in ADCs was restricted by narrow therapeutic indices [40].

Payloads targeting DNA topoisomerase 1

Over the past few years, amounts of researches had demonstrated that topoisomerase 1, which was involved in key regulatory pathways of cancers, could be a promising anticancer target for several malignancies [41]. Camptothecin and its analogs are a kind of potent topoisomerase 1 inhibitor and become the potential payloads of ADCs. DS-8201a, approved by Food and Drug Administration (FDA) recently, was an anti-Her2 ADC that conjugated the derivative of DX-8951f with trastuzumab [42]. And DX-8951f is a camptothecin analog that exerts higher antitumor activity than the other camptothecin derivatives [43]. In addition, sacituzumab and labetuzumab, which were ADCs designed to target TROP2 and CEACAM5 (carcinoembryonic antigen-related cell adhesion molecule 5), respectively, adopted 7-ethyl-10-hydroxycampthotecin (SN-38) as payloads [28]. SN-38 is semi-synthetic camptothecin and is the active metabolic of irinotecan which is famous as an antitumor prodrug [44]. Although SN-38 encounters some challenges such as high hydrophobic and limited effective conjugation sites, the approvement of Sacituzumab govitecan (Trodelvy) by FDA means SN-38 analogs should have satisfied the criteria for ideal ADC payloads.

Other novel payloads

In addition to the three types of payloads discussed above, several new payloads were investigated in the preclinical trials. For instance, MT-5111, a novel Her2-targeting engineered toxin body in clinical development which binds an epitope on Her2 distinct from trastuzumab or pertuzumab, used Shiga Toxin, a ribosomal inhibitor, as the payload [45]. BDC-1001, SBT6050, and NJH395 selected the Toll-like receptors (TLRs) agonist as the payload to stimulate a strong local antitumor immune response and these ADCs were assessed in patients with Her2-expressing solid tumors in phase I trials (Table 2) [46]. MM-302 was an ADC composed of a Her2-targeted antibody linked to the cytotoxic chemotherapy liposomal doxorubicin and the preclinical studies showed that its activity was better than that of anthracycline and liposomal doxorubicin, but it missed the endpoint in phase II Trials.

LINKAGE STRATEGIES BETWEEN ANTIBODY AND LINKER

The linkage strategies between antibodies and linkers are important considerations for the safety and efficacy of ADCs in terms of pharmacokinetics and pharmacodynamics [47]. To successfully bridge the antibody and the payload, some critical criteria have to be met for ADCs

construction. Firstly, linkers must be sufficiently stable to circulate in the bloodstream without premature cleavage, which can efficiently avoid the off-target toxicity of ADCs. Secondly, linkers also permit efficient releases of highly cytotoxic payload from the antibody molecules once the ADCs are internalized into the target cells. Thirdly, to suppress the aggregation of ADCs which presents a significant challenge for chemistry, manufacturing and controls (CMC), the hydrophobic properties of linkers [28] must be considered. In general, the naturally available amino acid residues, lysine, and cysteine on the antibody (Table 2) are commonly applied in the construction of ADCs approved or under clinical trials [48–51]. Currently, the prevalent conjugation strategies for anti-Her2 ADCs in the clinical trial are cysteine-conjugated (7 ADCs), such as RC-48 and SYD985, followed by lysine-conjugated (6 ADCs). In recent years, site-specific conjugation with engineered or natural amino acids on antibodies comes into the trend to afford homogeneous ADCs, taking ARX788 and A166 for example, respectively [52, 53].

Lysine conjugation linkage

The amines on lysine could react with activated carboxylic acid esters, typically N-hydroxy succinimides esters or penta-fluorophenol esters, which are water tolerated, to form amides (Fig. 2). These techniques are composed of the majority of the ADC conjugation method and have been successfully applied both in the first approved ADC by the US FDA, gemtuzumab ozogamicin, and the anti-Her2 ADC, T-DM1. However, a typical IgG1 antibody has 80 to 90 lysine residues, among which ~10 can be modified under forcing conditions. Conjugation on lysine residues results in heterogeneous ADCs with a drug-antibody ratio (DAR) from 0 to 8 and an average DAR from 3 to 4. Furthermore, the linker may attach to some lysine residues that are critical in antibody–antigen interactions, resulting in reduced binding affinity. This produces a heterogeneous mixture of several subspecies, some of which significantly impact antigen-binding properties and cytotoxicity, and pharmacokinetics of ADCs.

Cysteine conjugation linkage

The cysteine-conjugated approach is based on the reaction between cysteine residues of the antibody and specific thiols functional segments anchored on the linkers (Fig. 2). However, unlike lysine, there are scarcely free thiol groups in wild-type antibodies, which are mainly generated by reducing the disulfide bonds. IgG1 contains 4 interchain and 12 intrachain disulfide bonds for ADC construction. Fortunately, the 4 interchain disulfides, which are generally not critical for structural stability of IgG1, can be selectively reduced under mild conditions to give 2, 4, 6, or 8 free thiols while keeping the 12 intrachain disulfides intact [29]. Hence, compared with lysine-based conjugation, definite conjugation sites and the specific thiol reactions of cysteine conjugation linkage contribute to the superiority in controlled DAR and heterogeneity of ADCs. This conjugation method has been the major choice for ADC construction and used for brentuximab vedotin, polatuzumab vedotin,

and many other ADCs in clinical trials, as well as anti-Her2 ADCs, such as RC48 and SYD985. There are eight ADCs (Adcetris, Polivy, Padcev, Enhertu, Trodelvy, Blenrep, Zynlonta, and Tivdak) using cysteine conjugation among the 12 ADCs on the market.

Most of the ADCs have currently used similar cysteine conjugation features, such as thiosuccinimide linkage formed from thiols and alkyl maleimides. However, the generated thiosuccinimide adducts are now known to undergo retro-Michael additions and/or other thiol exchange reactions with proteins in the blood circulation, especially albumin. This could result in a decreased targeted cytotoxic activity as well as increased off-target toxicity. Using next-generation maleimide linkers such as selfhydrolyzing maleimide, maleimidephenylbutyl linker is one of the solutions to avoid retro-Michael additions and/or thiol exchange reactions. Site-specific conjugation as described below is another way to circumvent such reactions.

Site-specific conjugation based on engineered amino acid

Leveraging antibodies with engineered reactive amino acid residues to conjugate with linker payloads is a feasible method to obtain site-specific ADCs. These ADCs are characterized by uniform stoichiometry with DARs of 2 or 4 and show a better tolerated than traditional ADCs [28]. For instance, ADCs constructed using engineered cysteine technology (THIOMAB) could be highly homogeneous with a DAR of 2 (>90% homogeneity) [54]. And ADCT-502 and MEDI4276 are produced basing this technology. The difference is that ADCT-502 mutates six of eight cysteine residues involved in the formation of interchain disulphide bonds into serines, leaving two cysteines at position 220 of heavy chains for site-specific conjugation, whereas MEDI4276 mutates S239 and S442 in Fc region into cysteines for conjugation. Unfortunately, the clinical trials of the two ADCs have been discontinued, maybe because of intolerable toxicity. Another example of this technology refers to the incorporation of p-acetyl phenylalanine group into an anti-Her2 antibody to provide a keto group as a reactive site for the formation of a stable oxime with an alkoxyamine derivatized drug [55]. The ARX788 ADC, an anti-Her2-p-acetylphenylalanine- MMAF conjugate, inhibited xenograft tumor growth and is now in phase I/II clinical trial. The clearance and exposure parameters of this ADC were very similar to the corresponding naked antibody.

Another example of site-specific conjugation based on engineered amino acids is the enzymatic site-specific conjugation. This type of site-specific conjugation can also be implemented by using specific engineered amino acid tags on antibodies, which are specifically recognized by enzymes such as sortase A and transglutaminase. Sortase A derived from *Staphylococcus aureus* can only mediate transpeptidation between engineered LPXTG tag (Lys-Pro-X-Thr-Gly, X represents any amino acid) and oligoglycine compounds, and catalyze the transpeptidation reaction, which recognizes the motif Lys-Pro-X-Thr-Gly (X: any amino acid) and modifies C termini of the light and heavy chains of antibodies [56]. Then, cytotoxic drugs such as MMAE or maytansine can be anchored to the specific motif via an
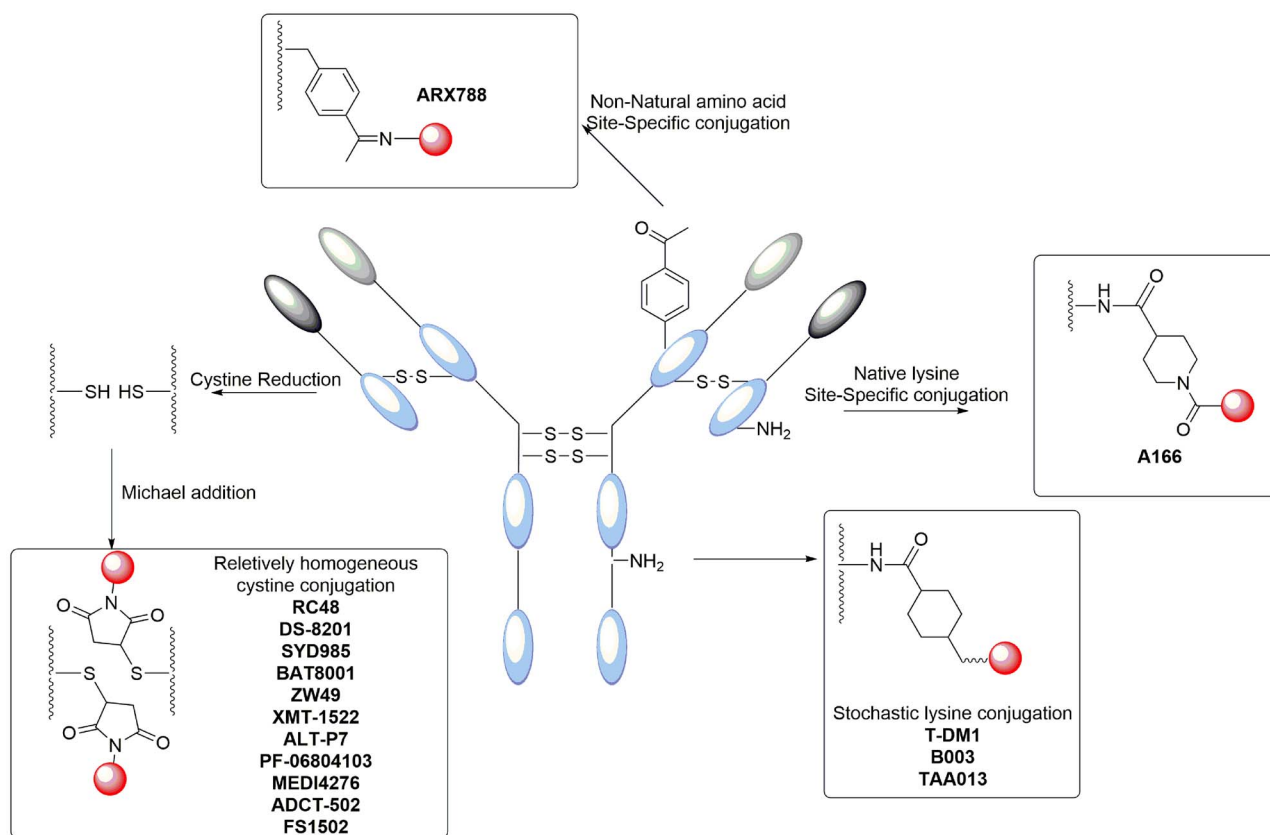
Figure 2. Schematics of four linkage formats between antibody and linker for Her2-targeted ADCs.

oligoglycine (oligo-G) [57]. In contrast, bacterial transglu-taminase derived from *Streptomyces mobaraensis* can mediate not only transpeptidation between engineered LLQG tag [58] and amine compounds but also native glutamine at 295 in deglycosylated or aglycosylated antibodies and amine compounds [29, 59, 60].

Site-specific conjugation of natural amino acid

The current site-specific conjugation techniques often require extensive protein engineering or are restricted to the N- or C-terminus, and thus limit their application. Great efforts have been made to generate chemo- and region-selective lysine modification on antibodies. Efforts have been made to generate homogeneous ADCs [61, 62]. A166, a homogeneous ADC, of which the linker is attached to the light chain on trastuzumab, is now in Phase I/II clinical trial [46]. Cysteine rebridging is a recently developed strategy that utilizes bis-alkylation conjugation at reduced cysteines derived from interchain disulfides, which can achieve many advantages in terms of better DAR, homogeneity, and stability of ADCs [63]. Furthermore, aryl palladium complexes that react with reduced cysteine residues of the antibody via thiol arylation function demonstrated the potential to directly conjugate trastuzumab and then attained a linker free ADC with average DAR of 4.4 [64]. The ADCs, although lacking a linker, showed equal binding affinity to recombinant Her2 than parent trastuzumab. With the significant advantages, such as stability under physical condition and controlled DAR, however, this strategy suffers the flaw of the toxicity of palladium.

RELEASE MECHANISMS OF THE PAYLOADS

Release strategies of the payloads are another key issue for ADCs development. Linkers must be stable to avoid premature release of payloads, whereas ADCs are circulating in the blood, but allow the efficient release of payloads from the antibodies after internalized into the target cells. Most of the second-generation ADCs in clinical trials were developed based on the maleimide-type linker, which suffered a deconjugation phenomenon in the circulation and causes off-target cytotoxicity [65]. Several strategies have been investigated to solve it in the third-generation ADCs. The release mechanism of payloads is usually categorized into cleavable and noncleavable one. Herein, we review the release mechanisms of payloads based on the anti-Her2 ADCs in the clinical trials.

The majority of ADC linkers adopted the cleavable linkers which can be conditionally cleared by an environment (such as low pH value) or by specific lysosomal enzymes (such as cathepsin B). Acid cleavable linkers, such as hydrazine, utilize the lower acidity of the endosomes and lysosomes than the blood circulation to retain the stable ADCs in the neutral pH but hydrolyze and release the cytotoxic drugs in acidic cellular compartments [66]. However, it has been associated with prominent toxicity and low tolerability under physiological conditions.

Disulfide linker is another prevalent cleavable linker and can be reduced by the high concentration of glutathione. This format provides more stable ADCs in the circulation than acid cleavable linkers and several candidates are in active clinical trials [67].

Dipeptide-containing linkers rely on a lysosomal protease, cathepsin B, which is overexpressed in a wide range of malignant cells and implicates the progression of the tumor [68]. Cathepsin B has carboxyl peptidase activity and selectively recognizes certain amino acid sequences to cleave a dipeptide linker on the C-terminal side of the sequences such as Phe-Lys, Val-Cit, Glu-Val-Cit, and GGFG [29, 69]. This kind of linker has been used in approved ADCs such as brentuximab vedotin and trastuzumab duocarmazine [37, 70]. Trastuzumab duocarmazine (Trastuzumab vc-seco-DUBA, SYD985) is hydrolyzed to deliver para-aminobenzyl alcohol (PAB)-Dimethyl Ethylenediamine (DMEDA)-seco-Duocarmycin hydroxyBenzamide Azaindole (DUBA), and then sequentially undergoes the self-elimination of PAB, DMEDA, and chlorine hydride (HCL) to release active DUBA (Fig. 3A) [71–75]. Trastuzumab deruxtecan (DS-8201a), is hydrolyzed to deliver NH2CH2-Dxd, and then endures the acid-induced self-elimination to release active Dxd (Fig. 3B) [76–78]. RC48 also utilizes cathepsin B to hydrolyze the dipeptide Val-Cit of ADCs to deliver PAB-MMAE, and then undergoes one step self-elimination of PAB to release the active MMAE (Fig. 3C) [50, 79, 80].

Some ADCs utilize noncleavable linkers to construct greater stable bindings between payload and antibodies than cleavable linkers. Noncleavable ADCs depend on the degradation of the antibody segments by proteolytic machinery and release payload molecules which are consist of the drug, linker, and an amino acid residue derived from the antibodies. Therefore, ADCs with noncleavable linkers usually have a more complex drug release mechanism that relies on the complete lysosomal proteolytic degradation of the antibody and a limited bystander effect due to the poor permeation with the amino acid appendage and is mainly used in the ADCs treating for hematological cancers or tumors with high antigen expression [66]. For instance, the first approved Her2-targeted ADC (T-DM1) with noncleavable MCC-DM1 has to release the payload Lys-MCC-DM1 through multi-step hydrolysis after internalized into the lysosome and Lys-MCC-DM1 cannot traverse the plasma membrane of neighbor cells (Fig. 3D) [81–83]. In addition, BAT8001, using noncleavable Mc-Maytansinoid (Fig. 3E), and ARX788, using the novel auristatin analog AS269 as payload by the site-specific conjugation with the non-natural amino acid, p-Acetyl phenylalanine (pAcF) (Fig. 3F) [84–86], suffered the similar drawbacks.

CLINICAL PROGRESS AND PERSPECTIVE

The clinical research data of anti-Her2 ADCs have been shown in Table 3, and it is not difficult to see that several anti-Her2 ADCs at the forefront of research and development such as DS-8201a, SYD985, RC48, and ARX788, similar to T-DM1, showed excellent antitumor activity in breast cancer treatment. Meanwhile, part of them showed

significant clinical benefits for other Her2-positive cancers. In a phase II clinical trial, DS-8201a attained notable improvements on therapeutic indexes (TIs)for patients with metastatic breast cancer, such as 60.90% objective response rate (ORR), 16.40 months progression-free survival (PFS), and 12 months overall survival (OS) [87]. Patients with Her2-positive gastric cancer also achieved clinical benefits from DS-8201a (ORR 43.2%, DCR (disease control rate) 79.5%, and PFS 5.6 months) [88]. In Her2-mutant non-small cell lung cancer, ORR was 72.7%, and median PFS was 11.3 months [89]. RC48 exhibited superior clinical benefits on urothelial cancer (ORR 51.2%, OS 13.9 months, PFS 6.9 months) [90], gastric cancer (ORR 18.1%, PFS 3.8 months, OS 7.6 months) [91], and breast cancer (ORR 36.7%, SD (stable disease) 60.0%, CBR (clinical benefit rate) 46.7%) [92]. In addition, DS-8201a and RC48 also showed significant efficacy in the treatment of patients with low Her-2 expression. DS-8201a attains an overall response rate (ORR) of 37% and a DCR of 87% in a phaseItrial for breast cancer with Her2 IHC^{1+} or IHC^{2+} [93]. RC48, in a multicenter phase II trial for Her2-negative urothelial carcinoma (IHC^{0} or IHC^{1+}), achieved an ORR of 25% and DCR of 75% [94]. Comparing the action mechanism of DS-8201a and RC48 with T-DM1 which failed to achieve the desired efficacy in gastric cancer and non-small cell lung cancer, the bystander effect elucidates the important difference between DS-8201a or RC48 and T-DM1 in payloads. The payloads (DXd and MMAE adopted in DS-8201a and RC48, respectively) can penetrate the cell membrane and kill adjacent Her2-low-expressing or Her2-negative cancers, whereas that of T-DM1, released the DM1 with an amino acid residue, shows poor bystander effect [95]. This may partially explain why T-DM1 has a poor TI compared with DS-8201a and RC48 in combating the diverse origins of Her2-positive cancers. It is worth noting that ARX788 showed significant efficacy in the treatment of patients with Her2-positive gastric cancer and PR was obtained in 9 (45.5%) out of 20 patients. In March 2021, FDA granted orphan drug designation to ARX788 for Her2+ gastric cancer. A166 using a lysine site-specific conjugation technology also showed a good antitumor effect in early clinical studies. For now, although the development of ADCs inevitably encountered frustration, for instance, BAT8001 was just terminated due to failing to meet the endpoint events in a phase III clinical trial, ADCs as targeted drug therapy are exhibiting excellent prospects for patients with various cancers.

CHALLENGES FOR ANTI-HER2 ADC DESIGN

The properties of the ADC molecule are determined by the balance of its components. For any given combination of mAb, linker, payload, site, type of conjugation, and the achieved DAR, the resulting products will consist of a mixture of ADC variants with different physico-chemical properties. This, in turn, depending on the resulting heterogeneity, may affect its stability, pharmacokinetics, and disposition, adversely changing the overall ADC safety and efficacy profile. Therefore, a well-selected integration of components is a crucial aspect for the success of an ADC. With the advances of ADC technologies, the design of ADC
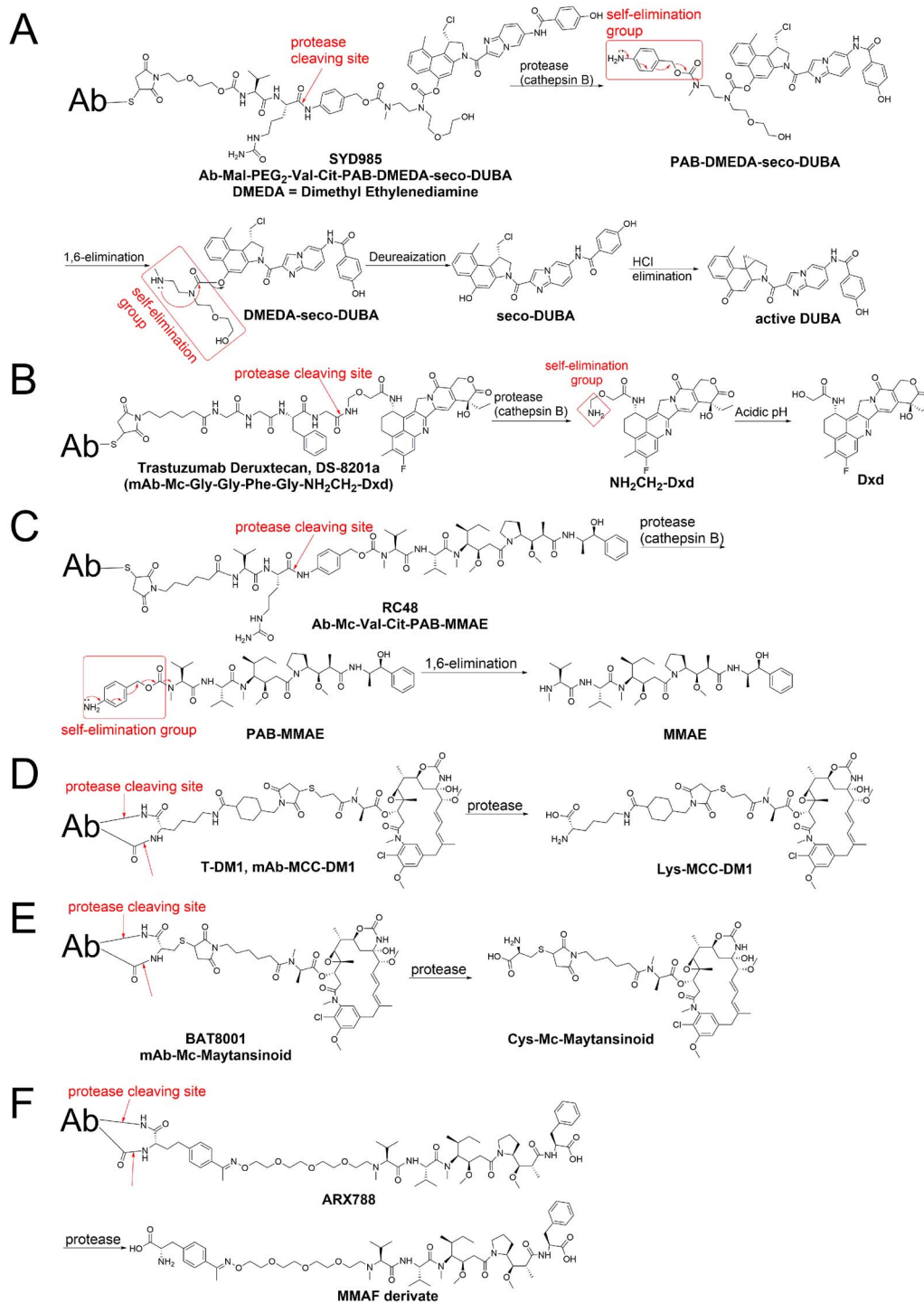
Figure 3. Six different payload release mechanisms of anti-Her2 ADCs. (A) The payload release mechanism of cleavable SYD985. (B) The payload release mechanism of cleavable DS-8201a. (C) The payload release mechanism of cleavable RC48. (D) The payload release mechanism of noncleavable T-DM1. (E) The payload release mechanism of noncleavable BAT8001. (F) The payload release mechanism of ARX788.

molecules is more diverse, which is both an opportunity and a challenge. In terms of antibodies, in addition to mAb, bispecific antibodies and Nbs can also be selected. Payload is not limited to cytotoxin, but also protein toxins, proteins, enzymes, radionuclides, ribosomal inhibitors, siRNA, and immunostimulants. There are also two types of linkers:

cleavable and noncleavable. In addition to the most common random, lysine or cystine conjugation, there are also a variety of site-specific conjugations including conjugation through engineered cysteine residues, non-natural amino acid, specific lysine, glycosyl, and enzyme-mediated conjugation. The DAR value can be as low as 2 or as high as 12.

Table 3. Summary of clinical data of anti-Her2 ADCs*

Name	DCR	PR	ORR	PFS (month)	OS (month)	Indication	Registry Trial ID	Phase
Trastuzumab emtansine (T-DM1)	-	-	-	9.6	30.9	Breast cancer	NCT00829166	3
Trastuzumab deruxtecan (DS-8201a)	-	-	60.90%	16.40	12	Metastatic breast cancer	DESTINY-Breast01	2
	79.5%	-	43.2%	5.6	-	Her2-positive gastric cancer	NCT02564900	1
			72.7%	11.3		Her2-mutant non-small cell lung cancer	-	1
Disitamab vedotin (RC48)	-	-	51.2%	6.9	13.9	Urothelial Cell carcinoma	NCT03507166	2
	-	-	18.1%	3.8	7.6	Gastric cancer	NCT03556345	2
	46.7%	-	36.7%	-	-	Breast cancer	NCT04400695	3
Trastuzumab duocarmazine (SYD985)	-	-	-	7.0	20.4	HER2-positive breast cancer	NCT03262935	3
MRG002	81%	43% (9/21)	43%	-	-	Solid tumors	NCT04941339	1
ARX788	91.7% (44/48)	39.6% (19/48)	-	-	-	Her2-positive breast cancer	CTR20171162	1a
	50% (10/20)	45.5% (9/20)	-	4.1	10.7	HER2-positive gastric cancer	CTR20190639	1
	100% (1.5 mg/kg)	-	67% (1.5 mg/kg)	-	-	Breast/gastric cancer	NCT03255070	1
BDC-1001	-	5% (1/20)	-	-	-	Solid tumors	NCT04278144	1/2
A166	86.4% (4.8 mg/kg) 85.7% (6.0 mg/kg)	-	59.1% (4.8 mg/kg) 71.4% (6.0 mg/kg)	-	-	Solid tumors	CTR20181301	1
	75% (6/8)	50% (4/8)	-	-	-	Solid tumors	NCT03602079	1
ALT-P7	77.3%(17/22)	13.3%(2/15)	-	-	-	Her2-positive advanced breast cancer	NCT03281824	1
TAA013	70%	-	10%	>5	-	HER2-positive breast cancer	CTR20181642	1

*Updated on 01 Nov 2021; PR: partial response.

Can the introduction of new technologies or new molecular design concepts certainly improve the success rate of ADC? The answer is unknown. For example, MEDI4276 is a biparatopic tetravalent antibody targeting two nonoverlapping epitopes in subdomains 2 and 4 (scFv) of the anti-HER2 mAb trastuzumab) of the HER2 ectodomain, with site-specific conjugation to a tubulysin-based microtubule inhibitor payload. The average DAR is 4. It demonstrates enhanced cellular internalization and cytolysis of HER2-positive tumor cells *in vitro*, but it displays intolerable toxicity at doses > 0.3 mg/kg in phase I clinical trials. XMT-1522 is an ADC that consists of a novel human IgG1 anti-Her2 mAb and a novel auristatin-based cytotoxic payload with a high DAR value [96]. The ADC is designed to remain stable in the bloodstream and to release the drug payloads once inside the targeted cell. But the company abandoned XMT-1522 after a disappointing risk-to-benefit ratio in a phase 1 study with breast cancer patients. Therefore, molecular design is still the key and difficult point in the development of ADCs.

CONCLUSION

In conclusion, the development of ADCs will benefit from the improvement of the designing of therapeutic mAbs to permit payloads conjugating at defined positions which achieve more homogeneous drug conjugates of ADCs. The diversification of linkage strategies and payload release mechanisms can provide more possibilities to generate ADCs with better efficacy and safety profile, actually, it has produced several potent anti-Her2 ADCs which not only effectively eliminated target tumors but also reduced the off-target toxicity to the normal tissues. The data of clinical trials showed, with the advances of ADC technology, the

TI of diverse ADCs had been significantly improved in the treatment of Her2-positive tumors.

ACKNOWLEDGEMENTS

FUNDING

CONFLICT OF INTEREST

The authors have declared no conflict of interests.

[JM F] holds the position of [Editor-in-Chief/Deputy Editor-in-Chief/Assistant Editor/Editorial Board Member] for Antibody Therapeutics and is blinded from reviewing or making decisions for the manuscript.

DATA AVAILABILITY

All figures generated during this study are included in this published article. All table data are available in Beacon targeted therapies, at https://www.beacon-intelligence.com/.

ETHICS and CONSENT STATEMENT

This is no applicable.

ANIMAL RESEARCH STATEMENT

This is no applicable.

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