REVIEW

Bispecific antibody drug conjugates: Making 1+1>2

Yilin Gu[a,†], Zhijia Wang[a,†], Yuxi Wang[a,b,*]

[a]*Targeted Tracer Research and Development Laboratory, Institute of Respiratory Health, Frontiers Science Center for Disease-Related Molecular Network, National Clinical Research Center for Geriatrics, West China Hospital, Sichuan University, Chengdu 610041, China*
[b]*Frontiers Medical Center, Tianfu Jincheng Laboratory, Chengdu 610212, China*

Abstract   Bispecific antibody−drug conjugates (BsADCs) represent an innovative therapeutic category amalgamating the merits of antibody−drug conjugates (ADCs) and bispecific antibodies (BsAbs). Positioned as the next-generation ADC approach, BsADCs hold promise for ameliorating extant clinical challenges associated with ADCs, particularly pertaining to issues such as poor internalization, off-target toxicity, and drug resistance. Presently, ten BsADCs are undergoing clinical trials, and initial findings underscore the imperative for ongoing refinement. This review initially delves into specific design considerations for BsADCs, encompassing target selection, antibody formats, and the linker−payload complex. Subsequent sections delineate the extant progress and challenges encountered by BsADCs, illustrated through pertinent case studies. The amalgamation of BsAbs with ADCs offers a prospective solution to prevailing clinical limitations of ADCs. Nevertheless, the symbiotic interplay among BsAb, linker, and payload necessitates further optimizations and coordination beyond a simplistic "1 + 1" to effectively surmount the extant challenges facing the BsADC domain.

*Corresponding author.

E-mail address: yuxiwang@scu.edu.cn (Yuxi Wang).

[†]These authors made equal contributions to this work.

1. Introduction

Antibody−drug conjugates (ADCs) comprise antibodies, linkers, and cytotoxic payloads[1]. Targeting tumors with ADCs is anticipated to mitigate systemic toxic effects compared to conventional chemotherapy, offering a broader therapeutic window and an elevated therapeutic index[2,3]. Over the last decade, ADCs have progressively matured in the treatment of both solid and hematological tumors, emerging as a revolutionary domain in anti-tumor drug research[4]. With the approvals of T-DM1 and DS-8201 for HER2+ breast cancer, the commercial clinical pipeline of ADCs has experienced steady growth[5,6]. Currently, more than 140 ADCs are in clinical development, with 15 having received approval.

While notable successes have marked ADC development, the emergence of unexpected toxicity, limited enhancements in toxicity profiles over the payload, and the onset of resistance pose formidable challenges. Various factors contribute to the prevailing clinical dilemmas, particularly in the context of solid tumors: (1) The heterogeneous nature of solid tumors constrains the clinical efficacy of targeting a singular antigen; (2) Therapeutic pressure induces antigen downregulation, epitope mutation, and the activation of bypass pathways, leading to substantial ADC resistance; (3) Factors such as target antigen expression in normal tissues, linker instability, and other variables contribute to imprecise payload release, causing off-target toxicity; (4) The resistance of the target antigen to internalization impedes a sufficiently lethal effect[2,7]. These unresolved issues impede the clinical advancement of ADCs. Strategic optimizations of antibodies, linkers, and payloads emerge as a promising avenue for the next-generation ADCs, holding the potential to surmount current clinical challenges.

The predominant source of ADC toxicity lies in the linker−payload complex, while the binding of antibodies to antigens also exerting a substantial influence on drug effects[8]. A prospective approach to address the aforementioned clinical challenges involves the conjugation of bispecific antibodies (BsAbs) to linker−payload complex, giving rise to the concept of bispecific antibody drug conjugates (BsADCs) (Fig. 1). BsAbs are antibodies binding to two distinct epitopes on either the same or different antigens[9,10]. Presently, over 400 different forms of BsAbs are undergoing phase III clinical trials, with ten having received approval[11]. In contrast to traditional ADCs, the unique dual epitope/target binding modes of BsADCs not only enable binding to co-expressed antigens in solid tumors to enhance selectivity but also significantly improve internalization[12,13]. These distinctive advantages position BsADCs as a substantial force in the realm of next-generation ADCs. Currently, ten BsADCs are undergoing clinical trials (Table 1), with some early clinical trial results presented at the recent AACR and ESMO conference. Despite advancements, the safety and efficacy fall short of theoretical expectations, underscoring that the design of BsADCs is not merely "1 + 1". Alterations in the binding patterns of BsADCs impact the overall efficacy, emphasizing the necessity for comprehensive coordination and optimization of BsAbs, linkers, and payloads. Persistent challenges faced by BsADCs include limited antibody scaffolding, heterogeneous coupling orientations, inconsistent bispecific antibody generation, unknown parameters between two targets, and potential safety concerns. This review systematically encapsulates the design strategies of BsADCs, encompassing composition, design considerations, and current advancements and challenges, with the aim of paving the way for design of the next-generation ADCs.
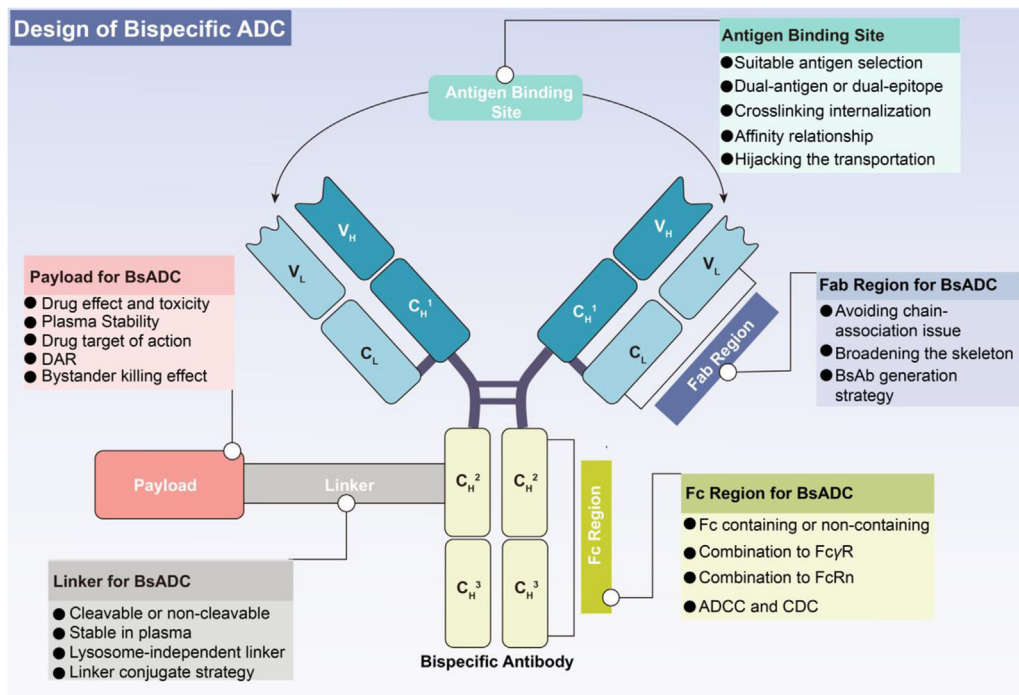
2. Design considerations on BsADCs

The current repertoire of known BsAb skeletons for BsADCs remains relatively confined, with targets primarily focused on HER2, EGFR, and c-MET. Despite the abundance of BsAbs, there is a need for further exploration to ascertain their suitability for constructing BsADCs[14]. The modular components of ADCs—antibody, linker, and payload—require independent optimizations, particularly when considering distinct tumor subtypes[7]. Minor modifications to any of these key components can yield substantial alterations in clinical characteristics. Merely substituting antibodies with BsAbs is insufficient for fully characterizing applicable BsADCs[15,16]. In designing future BsADCs, the replacement of antibodies, optimizations of the linker−payload complex, and conjugation strategies should be viewed as interconnected networks, necessitating a holistic approach.

2.1. Design considerations on targets of BsAbs

The paramount considerations in the construction of BsADCs lie in the judicious selection of a suitable target combination. Target selection serves as a fundamental prerequisite for the successful development of ADCs, exerting a pivotal influence on the ultimate therapeutic window and systemic toxicity. Given the prevalent challenges of general off-target toxicity and clinical resistance faced by traditional ADCs, the following criteria should guide target selection:

(1) The conventional guiding principle involves identifying antigens characterized by good internalization, relatively low expression on normal tissues, and high expression on tumors[17]. (2) Acknowledging the heterogeneity of solid tumors, it becomes imperative to ascertain the expression levels of targets in various tumor subtypes and sites, facilitating the optimal implementation of tailored drug delivery rather than relying on a singular "magic bullet" approach[18]. (3) Owing to the unique dual targeting characteristics of BsADCs, a comprehensive consideration of the deep-level effects of antigen combinations is essential. This encompasses factors include internalization, recycling, rates of turnover, lysosomal degradation, and obligate mechanisms[7]. The thoughtful integration of these considerations is indispensable for effective BsADC design, ensuring a nuanced understanding of the dynamic interplay between antigens and their cellular processes.

Following target binding, the internalization of the ADC−antigen complex stands as the pivotal process determining drug delivery. The types of antigens, properties of the antibody, and binding modes collectively influence the internalization of ADCs and the pace at which it occurs[19]. Critical antibody properties, such as valency, shape, size, isoelectric point[20], and hydrophobicity[21], exert a profound impact. Additionally, the binding mode and site further shape the internalization process. For instance, in the study by Hung et al., ten PD-L1 antibodies were designed to identify glycosylation sites, with only those recognizing N192 and N200 demonstrating internalization[22]. This implies that, even when targeting the same antigen, the internalization of ADCs may range from excellent to unacceptable. BsADCs offer an effective avenue for enhancing internalization through obligate mechanisms (e.g., hijacking and antigen aggregation). The rapid turnover targets can markedly enhance internalization speed, even in cases of lower expression on tumors (e.g., HER3[23] and Prolactin receptor[24]). Leveraging fast-turnover receptors as BsADC targets can significantly improve overall internalization effects[25]. Furthermore, the unique dual

Figure 1 The concept and design considerations of BsADCs. (1) Antigen binding site: critical considerations for the antigen binding site encompass the selection of antigen combinations, the binding model, crosslinking internalization, and cellular transportation; (2) Fab and Fc region: pertinent issues in the Fab and Fc region include addressing chain-association challenges, BsAb generation, broadening of the antibody skeleton, and exploring combinations with FcγR and FcRn; (3) Linker−payload complex: further optimizations are warranted for the linker−payload complex to enhance its effectiveness in the BsADC design.

binding mode of BsADCs promotes the formation of larger antigen−antibody complexes on the membrane, thereby inducing substantial endocytosis and target downregulation[26]. This nuanced understanding of internalization dynamics underscores the potential of BsADCs to optimize drug delivery through strategic design considerations.

Table 1 BsADCs in clinical trials. Data source: Clinicaltrials.gov.

Type	Drug	Target	Payload	Phase	Year[a]	Country	Company	Status	NCT number
BsADCs on HER2	ZW-49	HER2 × HER2	*N*-Acyl sulfonamide auristatin	I	2019	U.S.	Zymeworks Co., Ltd.	Active, not recruiting	NCT03821233
	MEDI4276	HER2 × HER2	Tubulysin Warhead	I	2015	U.S.	AstraZeneca Co., Ltd.	Completed	NCT02576548
	JSKN003	HER2 × HER2	TOPIi	I	2022	Australia	Alphamab Co., Ltd.	Recruiting	NCT05494918
				I	2023	China		Recruiting	NCT05744427
	KM501	HER2 × HER2	Microtubule inhibitor	I	2023	China	Xuanzhu Co., Ltd.	Not yet recruiting	NCT05804864
	TQB2102	HER2 × HER2	TOPIi	I	2023	China	ChiaTai TianQing Co., Ltd.	Not yet recruiting	NCT05735496
BsADCs on EGFR	AZD9592	EGFR × c-MET	TOPIi	I	2022	U.S.	AstraZeneca Co., Ltd.	Recruiting	NCT05647122
	M1231	EGFR × MUC1	Hemiasterlin	I	2021	U.S. Canada	EMD Serono	Completed	NCT04695847
	BL-B01D1	EGFR × HER3	TOPIi	I	2022	China	Baili Co., Ltd.	Recruiting	NCT05470348
				II	2023	China	Baili Co., Ltd.	Recruiting	NCT05924841
				III	2023	China	Baili Co., Ltd.	Recruiting	NCT06118333
BsADCs on MET	REGN5093-M114	MET × MET	Maytansinoid payload	I/II	2021	U.S.	Regeneron Co., Ltd.	Recruiting	NCT04982224
BsADCs on FRα	IMGN151	FRα × FRα	Tubulin inhibitor, DM21	I	2023	U.S.	ImmunoGen, Inc.	Recruiting	NCT05527184

[a]Actual study starting date. TOPIi, topoisomerase I inhibitor; MMAE, monomethyl auristatin E; Co., Ltd., company limited; Inc., incorporated.

Rapid turnover receptors and the formation of large antigen–antibody complexes can redirect the internalized complex to lysosomes rather than recycling, as observed in certain contexts[25]. Subsequent to internalization, ADCs must traverse the endosome to reach the lysosome for efficient payload release[27,28]. Treatment pressure from ADCs can alter *trans*-endosomal transportation, resulting in decreased lysosomal delivery and increased distribution to other cell compartments or enhanced recycling[27,28]. This not only diminishes efficacy[29] but also fosters drug resistance to ADCs[30,31].

The ADC–antigen complex can undergo internalization through various mechanisms, including Fc-mediated non-specific endocytosis[32], clathrin-mediated endocytosis[33,34], caveolin-mediated endocytosis[33], and pinocytosis[21,29,35,36]. Of these, clathrin-mediated endocytosis emerges as the predominant route for ADC internalization. In the context of BsADCs, they are directed to the sorting endosome and the endocytic recycling compartment during clathrin-mediated endocytosis, a process regulated by small GTP-binding proteins of the RAB and ARF (ADP-ribosylation factor) families. This intricate interplay of internalization mechanisms underscores the importance of understanding and optimizing the endocytic journey of BsADCs for effective drug delivery and to mitigate the risk of resistance (Fig. 2A)[37,38]. Commencing from the early endosome (pH = 6.0–6.5), rapidly recycling receptors (*e.g.*, EGFR[39,40] and TGF-βR[41]) typically undergo recycling to the cell membrane through RAB4-dependent fast recycling and RAB8 and RAB11-dependent slow recycling[38,42]. Conversely, fast-turnover receptors such as PRLR[24] are transported through a RAB7-dependent degradative route into late endosomes (pH = 5.0–6.0), followed by lysosomes (pH < 5)[38,42]. The fate of the internalized complex—whether it is recycled or directed to lysosomes for degradation—hinges on the interaction of specific sequences (motifs) in the cytoplasmic domains of proteins with elements of the coated pits[42]. Membrane receptors containing ubiquitination signaling and YXXØ consensus motifs can recruit clathrin and the clathrin adaptor protein 2 (AP2) to the plasma membrane, with AP2 discerning and facilitating transportation to lysosomes.

To address the decline in efficacy and drug resistance arising from the redirection of the internalization pathway, one strategy involves designing BsADCs that degrade in weakly acidic environments (sorting endosome, the endocytic recycling compartment, and late endosome) before reaching lysosomes to release the payload[28,43]. Another approach is to select membrane receptors containing the YXXØ motif (*e.g.*, CD63[44] and APLP2[45]), ubiquitination signaling (*e.g.*, PRLR[46]), or other lysosomal sorting signals as one of the targets. Early endosomal processing and lysosomal hijacking render receptors with recycling potential or poor internalization as viable targets for BsADCs, significantly expanding the range of options for ADC targets. The complete internalization transportation process of ADCs, particularly in non-clathrin-mediated endocytosis, remains inadequately understood, highlighting the need for further in-depth research.

The properties of the antibody and the antibody–antigen binding interaction demand meticulous consideration[26]. In comparison to conventional ADCs, BsADCs, with their bispecific binding properties, introduce obligate mechanisms (Fig. 3C). This confers a decisive advantage by simultaneously blocking two (or more) targets, surpassing the capabilities of conventional ADCs and expanding the therapeutic landscape from traditional chemotherapy to blended therapy[47]. Additionally, bispecific targeting facilitates passage through biological barriers. For instance, targeting transferrin receptor[48] and Ephrin A2 (EphA2)[49] can mediate effective transcytosis, presenting a promising avenue for constructing BsADCs targeting brain tumors through the blood–brain barrier[50,51].

Bispecific targeting properties play a pivotal role in overcoming multi-mechanism drug resistance arising from poor internalization, down-regulation, binding site mutation, and the activation of bypass pathways[52,53]. To promote antigen aggregation on the cell surface, structural analysis is imperative. The distance between two binding sites and epitopes should be carefully considered to prevent binding on the same antigen. T-cell redirecting BsAbs have the potential to redirect T-cells to the tumor microenvironment (TME), opening avenues for designing T-cell redirecting BsADCs[54]. This has the potential to significantly broaden immunotherapy options, particularly in the treatment of hematological tumors[55,56]. The heterogeneity of solid tumors poses challenges in accurately targeting both T cells and tumors for both arms. Moreover, BsADCs targeting non-internalizing targets (*e.g.*, TME and immune cells) warrant attention with regards to drug release. The design of non-internalizing BsADCs utilizing cleavable linkers and permeable payloads emerges as a promising strategy.

2.2. Design considerations on formats of BsAbs

Antibodies consist of two heavy chains and light chains, encompassing the Fc region and Fab region. When constructing ADCs, the fundamental requirements for the antibody skeleton include a suitable circulating half-life and low immunogenicity[57]. One of the primary classification criteria for BsADCs is whether they contain the Fc region[14,58]. Absence of the Fc region results in antibodies either comprising the variable VH and VL domains of two antibodies or being based on Fab fragments[59,60]. The construction is more convenient due to the absence of the Fc region, and the smaller molecular weight enhances penetration[61]. Meanwhile, the loss of binding ability to FcγR reduces Fc-mediated nonspecific drug uptake[62,63]. Conversely, the loss of binding to the neonatal Fc receptor (FcRn) leads to a relatively short half-life in plasma[64] (Fig. 2B). The design of Fc non-containing BsADCs faces challenges such as low stability, aggregation issues, and lack of coupling sites. On the other hand, Fc-containing BsADCs bring additional advantages such as antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), immune phagocytosis, and cytokine release, which collectively contribute to tumor killing[65]. However, the binding to FcγR during endocytosis may lead to the internalization of BsADCs in normal tissues, resulting in off-target toxicity[62,63]. In summary, strategies for Fc region construction include: (1) Fc engineering: This involves modifications such as amino acid modification in Fc region[66], mutation introducing[67,68], and glycosylation modifications[69]. These approaches can help mitigate off-target toxicity caused by binding to FcγR; (2) Retain the ADCC and CDC: The dual-target binding mode is conducive to hexamer formation and can enhance the CDC effect. And ADCC could improve the tumor killing effect; (3) Retaining FcRn binding or applying antibody engineering helps improve half-life and safety[70].

Additionally, the generation of BsAbs for BsADC design is also crucial. This aims to avoid chain-association issues[71], simplify the production process, reduce development costs, and improve product stability. Further evaluation and exploration in this aspect are warranted, and recent reviews provide detailed insights into this area, which we will explore in the perspective section[14,64,72].
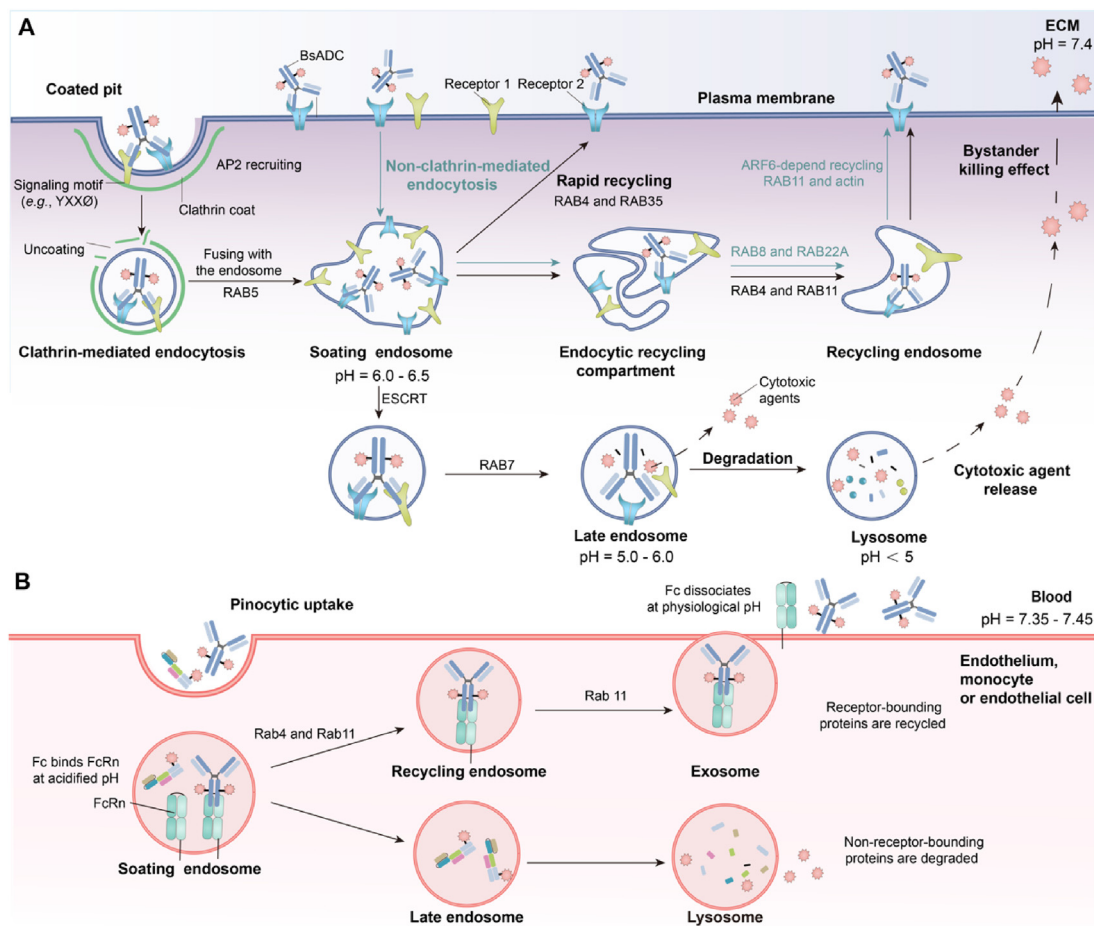
Figure 2 (A) BsADCs possess a distinctive advantage in promoting internalization and lysosomal degradation, a process involving both clathrin-mediated endocytosis and non-clathrin-mediated endocytosis. (1) ADCs targeting fast-turnover receptors (light green receptor in Fig. 2A) with signaling motifs (*e.g.*, YXXØ) can undergo internalization through clathrin-mediated endocytosis (black arrow). The endocytosed antigen−antibody complex, guided by RAB5, enters the sorting endosome (also known as the early endosome, pH = 6.0−6.5). Subsequently, it may enter the recycling process through RAB4 and RAB11-mediated recycling, or opt for the RAB4 and RAB35-mediated rapid recycling process to the cell membrane surface (pH = 7.4). (2) Alternatively, ADCs can also be internalized through non-clathrin-mediated endocytosis (green arrow). (3) In sorting endosomes, antigen−antibody complexes are sorted into late endosomes *via* ESCRT mediation. The complex then proceeds to the lysosome (pH < 5) guided by RAB7, where the ADC undergoes degradation, releasing the payload. It's important to note that the pH values and RAB associations provided are general, and actual values may vary based on cell types; (B) The Fc binding to FcRn is crucial for the blood stability of ADCs. When the ADC is taken up by vascular endothelial cells or monocytes, the Fc segment within the ADC engages with FcRn, leading to its recycling back into the extracellular space. This recycling process helps prevent early degradation, contributing to the blood stability of ADCs.

BsADCs can be classified based on their binding patterns, involving either simultaneous binding to two different antigens (dual-antigen binding mode) or binding to two different epitopes on the same antigen (dual-epitope binding mode) (Fig. 3A). These two binding modes profoundly impact the pharmacodynamics (PD) and pharmacokinetic (PK) properties of BsADCs[73]. The dual-antigen binding mode not only enhances selectivity but also has the potential to block signaling pathways, overcome drug resistance, and provide a synergistic killing effect[74]. Specific target selection in this mode can improve internalization, drug effects, and reduce drug resistance. Targeting fast turnover receptors, such as PRLR, may enhance internalization and hijack lysosomal transportation. Constructing BsADCs targeting c-MET and EGFR, for instance, can effectively overcome drug resistance

associated with EGFR monotherapy[74−78]. To develop promising dual-target BsADCs, careful regulation of the affinity ratio for the two targets is essential, ensuring simultaneous binding without triggering target activation[79]. Further analysis is required to determine the expression thresholds of targets to ensure effective tumor killing. It is therefore necessary to characterize positive antigens on cancer cells more precisely to inform future clinical applications.

2.3. Design considerations on linker−payload complex

The linker in BsADCs serves as the crucial connection between the antibody and the cytotoxic payload, playing a vital role in payload release and drug stability[80]. An ideal linker should exhibit
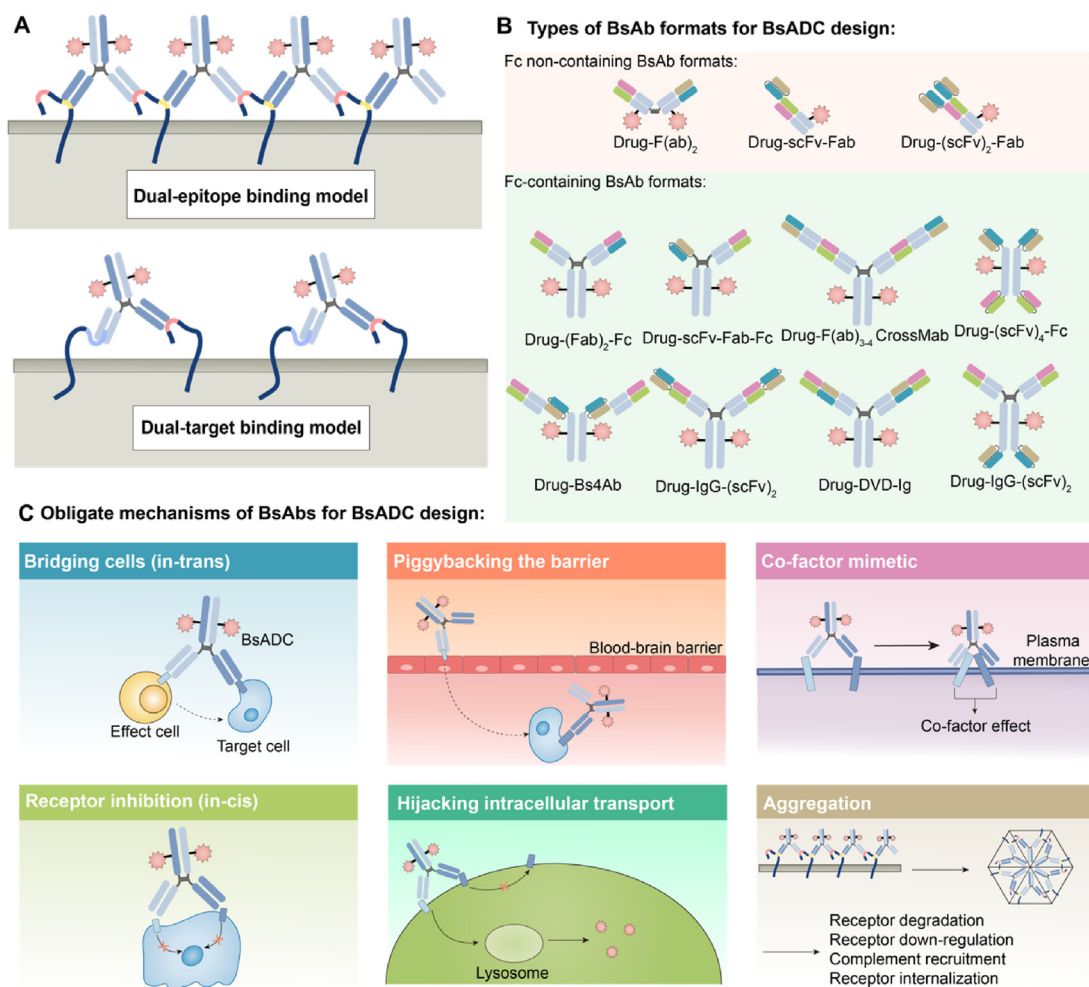
Figure 3 Design considerations on types of BsAb in BsADCs. (A) Dual-epitope and dual-target binding model of BsADCs; (B) Types of BsAb formats for possible BsADC design; (C) The dual-targeting mechanism of BsAbs provides BsADCs with obligate mechanisms.

stability in the plasma while facilitating effective release in the tumor. Presently, linkers in ADCs can be categorized into cleavable and non-cleavable linkers.

Non-cleavable linkers, characterized by high stability in plasma, can only undergo degradation in the lysosome to release the payload. This type of linker leads to lower off-target toxicity, increased plasma half-life, and enhanced safety. However, potential drug resistance may arise from impediments to internalization and lysosomal transport[81,82]. BsADCs based on non-cleavable linkers should focus on further optimizing internalization, as well as subsequent endosomal transport and lysosomal degradation[83,84].

Compared to non-cleavable linkers, ADCs based on cleavable linkers find wider applications. Cleavable linkers control payload release primarily by leveraging microenvironmental differences between body circulation and tumors[80]. The major challenge for cleavable linkers lies in off-target toxicity resulting from non-specific release. Cleavable linkers can be categorized as chemical cleavable linkers (*e.g.*, acid[85] and GSH-cleavable linkers[86,87]), enzyme cleavable linkers (*e.g.*, cathepsin cleavable linkers[88,89]), and others (*e.g.*, photo-responsive[90,91] and bioorthogonal cleavable linkers[92,93]). To design lysosome-independent BsADCs, certain cleavable linkers (*e.g.*, Val-Cit, VC linker) can facilitate payload release effectively in early and late endosomes[30]. There is evidence

indicating the existence of chemical or enzymatic cleavage triggers extracellularly, leading to the proposition of non-internalizing ADCs in recent years. Non-internalizing ADCs can target antigens in TME and vascular system, activating a bystander killing effect[94,95]. By utilizing cleavable linkers and bystander payloads, there is potential for designing non-internalizing BsADCs[96].

Emerging strategies in linker and conjugation methods shape the design of antibody−drug conjugates. The Val-Cit-*p*-aminobenzyl carbamate linker, widely used in BsADC design, demonstrates acceptable stability but is associated with dose-limiting adverse effects hindering clinical application[97]. Addressing this, the introduction of polar residue substitutions and mimetic peptides in the VC linker enhances plasma stability and lysosomal cleavage ability[98,99]. Ongoing efforts focus on exploring novel linkers, such as legumain-sensitive[100] and tandem cleavable linkers[101], to achieve sufficient drug release in targeted cells and improved aqueous solubility. Promisingly, the identification of novel enzyme candidates like α-l-iduronidase, with over-expression in specific tumor types and high cleavage efficiency, is underway[102,103]. Beyond linker innovation, comprehensive discussions on the chemical and structural dynamics of conjugation sites play a vital role in shaping the properties of ADCs[104].

Cytotoxic payloads, integral to BsADCs, play a crucial role in determining the overall antitumor effect and potential adverse

reactions on a significant scale. Ideal payloads for ADCs need to exhibit high drug effects at nanomolar to picomolar levels, considering the low permeability of ADCs[105,106]. Additionally, these payloads should possess sufficient plasma stability, low immunogenicity, and appropriate water solubility[107]. Finally, the payload should feature an available group for coupling to the antibody. ADC payloads encompass tubulin inhibitors, DNA/RNA damaging agents, immune-targeting agents, and novel payloads. Advances in ADC payloads have been summarized in our recent review. Further considerations in constructing the linker−payload complex are essential for designing ideal BsADCs:

The primary consideration involves choosing the appropriate conjugation method and drug antibody ratio (DAR), representing the number of linker−drugs attached to a given antibody. There are substantial differences in the *in vivo* pharmacokinetics of various drug-carrying forms of ADCs (DAR = 0−8). For BsADCs, the DAR typically ranges between 2 and 4, striking an optimal balance between kidney clearance and potency[16]. In cases where drugs with lower toxicity, such as using PROTACs[108] and topoisomerase inhibitors[109,110], higher DAR values may be necessary to achieve sufficient cell-killing effects. However, a simple increase in payload conjugation at high DAR can lead to conjugate hydrophobicity, resulting in aggregation, fast clearance, and poor efficacy[111]. Additionally, incomplete coupling of the payload with antibody residues, especially at high DAR, can contribute to conjugation heterogeneity, complicating characterization and posing potential toxicity issues[112,113]. Traditional non-specific derivatization of BsAbs surface lysine residues not only induces conjugation heterogeneity but also has the potential to influence dual-target binding effects. Homogeneous conjugation strategies, such as site-specific conjugation technologies[114] and enzyme-based conjugation methods[115], can be applied to produce uniform and purifiable BsADCs. These methods minimize the potential impact of drug coupling on dual-target binding[116].

The bystander killing effect of payloads in BsADCs is a crucial aspect deserving further discussion. Bystander killing effect refers to the ability of payloads to kill adjacent untargeted cells after release. It is a double-edged sword for the PK/PD properties of ADCs[117−119]. While the bystander killing effect can enhance the overall efficacy of ADCs in the heterogeneous tumor environment, it also poses a risk of off-target killing in normal tissues around the tumor[118]. This effect relies on cleavable linkers and hydrophobic payloads[119]. If one of the target antigens has a certain level of expression in normal tissues, such as c-Met, BsADCs should avoid applying payloads with bystander effects[120]. As mentioned earlier, the bystander killing effect of payloads enables the construction of non-internalizing BsADCs[95,121]. In summary, the bystander killing effect holds promise for BsADCs in overcoming tumor heterogeneity, the tumor barrier, and poor internalization. However, potential safety issues associated with off-target effects need to be carefully addressed.

ADCs face challenges related to dose-limiting toxicities, highlighting the need for innovative payloads. Novel drug payloads are crucial for BsADC development. By linking POI and E3 ligase, PROTAC induces ubiquitination and subsequent proteasomal degradation of POI, enabling effective target protein degradation at lower doses—making PROTACs ideal payloads for BsADCs[122]. Antibody−PROTAC conjugates, or DACs, show promise in enhancing PROTAC delivery efficiency *in vivo*. Bispecific DACs hold potential for diverse applications in targeted protein degradation. Besides, immune-stimulating payloads for

autoimmune and inflammatory diseases, such as Toll-like receptor agonists[123], STING agonists[124], and glucocorticoid receptor modulators[125], are also promising[126]. Additionally, emerging payloads like ferroptosis inducers[87], oligonucleotides[127], and DNA frameworks[128] contribute to the expansion of drug options in the BsADC field. The development of novel drugs can significantly enrich the types of optional drugs in the field of BsADCs.

3. Current advances and issues on BsADCs

3.1. BsADCs on HER2

HER2 overexpression is observed in approximately 20%−25% of breast cancer patients through techniques like Immunohistochemistry and fluorescence *in situ* hybridization, classifying them as HER2-positive. Clinically approved HER2-targeting monoclonal antibodies (mAbs) and ADCs, such as T-DM1 and DS-8201, are designed to hinder invasive tumor growth[129−131]. HER2-targeted therapy has significantly transformed treatment options for HER2-positive breast cancer, yielding clinical benefits. However, certain clinical challenges persist[6]. The expression heterogeneity of HER2 poses a limitation to the efficacy of current anti-HER2 therapies in cases of relatively low HER2 levels. This not only narrows the range of indications for HER2 therapy but also fosters drug resistance under therapeutic pressure[81]. Additionally, the potential resistance of HER2 to internalization diminishes drug efficacy. In summary, pharmacokinetic limitations, intratumoral heterogeneity, and the absence of bystander effects impede the advanced clinical application of HER2-targeted drugs[132].

In addition to breast cancer, HER2 overexpression can occur in other solid tumor subtypes, such as gastric cancer, non-small cell lung cancer, and colon cancer. However, clinical trials have shown that HER2-targeted drugs do not significantly improve the prognosis of HER2-positive patients with these cancers[133,134]. For instance, a phase III trial revealed that pertuzumab plus trastuzumab and chemotherapy for HER2-positive metastatic gastric or gastro-oesophageal junction cancer did not lead to a significant improvement in survival and clinical benefits[135]. This lack of improvement may be attributed to the relatively low HER2 expression in other solid tumor subpopulations compared to HER2-positive breast cancer. Consequently, poor internalization and differentiation mechanisms may contribute to insufficient clinical benefits. Fortunately, the dual binding model of BsADCs offers a promising solution to the current challenges of poor internalization and drug resistance in HER2-targeting for breast cancer. BsADCs have the potential to enhance anti-tumor activity in other tumor subtypes by improving internalization effects and exhibiting favorable PK/PD characteristics (Fig. 4).

3.1.1. Dual-epitope BsADC targeting HER2
ZW49. To address potential issues of poor efficacy and clinical drug resistance in HER2-targeted therapy, the biparatopic HER2 antibody Zanidatamab has been characterized[136]. Zanidatamab enhances the anti-tumor effect through various mechanisms, including target antigen neutralization, activation of cell-mediated cytotoxicity, and CDC. By introducing mutations into the Fab segment of the extracellular domains 2 (ECD2) binding domain, its unique dual epitope binding mode can effectively induce target antigen cross-linking, resulting in receptor-driven internalization and increased
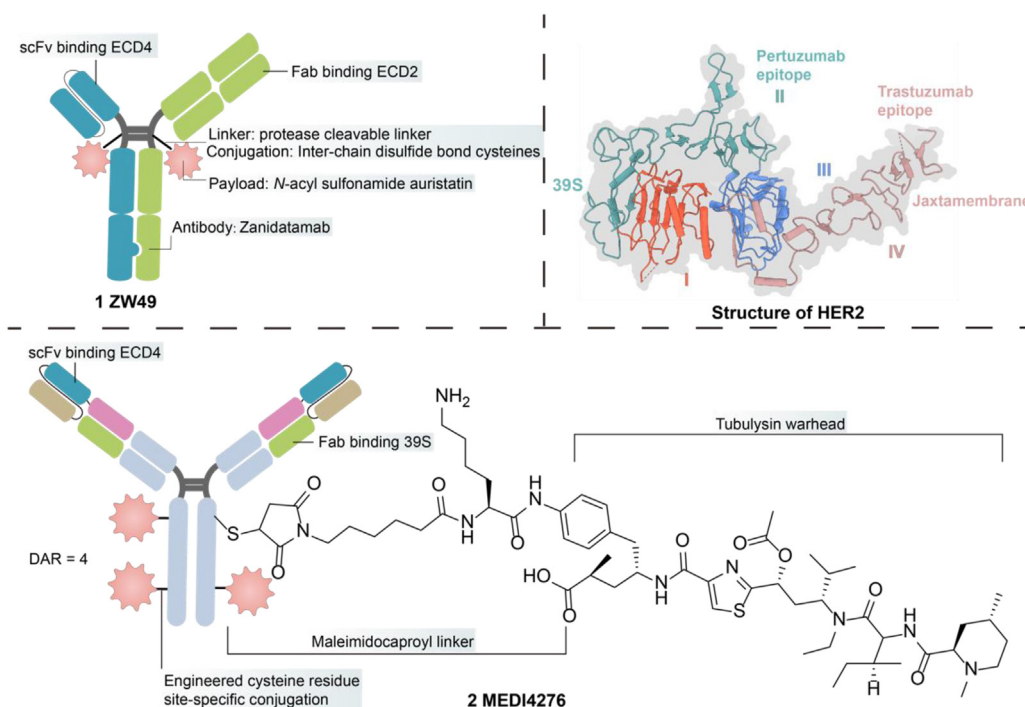
Figure 4 Structure of ZW49 and MEDI4276; 39S and trastuzumab epitopes are located at opposite ends of HER2 molecule (more than 90 Å). The linker employed in MEDI4276 can hardly cover the distance of 60 Å, which prevents binding simultaneously to the same antigen molecule (PBD id: 3BE1).

receptor downregulation. By using the IgG1-like heterodimeric Azymetric™ Fc platform, an anti-HER2-ECD4 single-chain variable fragment (scFv) was linked to heavy chain 1, and an anti-HER2-ECD2 Fab domain was linked to heavy chain 2[136,137]. The scFv and Fab arms of the original precursor biparatopic molecule bound to HER2 ECD with a K_D of 1 and 15 nmol/L, respectively[136]. Mechanism analysis reveals that Zanidatamab can bind two HER2 *via* a trans receptor binding model, inducing the formation of large, polarized aggregate clusters. This not only improves the internalization effect but also promotes antibody hexamerization, achieving enhanced CDC effects[136]. Zanidatamab has progressed through phase I and phase IIb trials, demonstrating favorable clinical benefits[138,139]. Its characteristics as an excellent internalized antibody with a potent tumor-killing effect make it conducive to further design as the antibody component of BsADCs. An anti-HER2 biparatopic ADC, ZW49, has been designed based on Zanidatamab, utilizing inter-chain disulfide bond cysteines and a protease-cleavable linker[140]. ZW49 incorporates a novel payload, *N*-acyl sulfonamide auristatin, making it well-tolerated. The bispecific antibody nature of ZW49 contributes to superior internalization compared to trastuzumab. Its Fc region imparts ADCC, ADCP, and CDC effects, while the hexamer mode of HER2 enhances CDC and internalization[140]. This design addresses several unmet clinical needs for patients expressing HER2. Preclinical data indicates that ZW49 exhibits a potent tumor-killing effect and good tolerance without compromising HER2 affinity (highest non-severely toxic dose = 18 mg/kg). Currently, ZW49 is undergoing a phase I clinical trial. As of September 2022, ZW49, the first BsADC to disclose clinical trial data, has demonstrated superior internalization effects. The objective response rate (ORR) in patients with advanced HER2-expressing solid tumors is 31%,

although its ocular toxicity characteristics (keratitis in 42%) cannot be ignored[141].

MEDI4276. T-DM1 and DS-8201 face efficacy challenges in cases of low HER2 expression due to resistance to internalization. The bispecific binding mode of BsADCs holds promise for enhancing intracellular drug delivery, overcoming resistance, and broadening applicability across a wider range of cancer cell populations[52]. The antibody 39S, derived through hybridoma technology, targets a distinct epitope on HER2 compared to trastuzumab. By utilizing the variable domain sequences of 39S and trastuzumab, a biparatopic antibody was constructed, incorporating the scFv of trastuzumab at the N terminus of the heavy chain of 39S IgG1. This BsAb retains HER2 binding specificity and benefits from the synergistic effect of both arms, effectively blocking HER2 homo- or heterodimerization[142]. Structural analysis indicates a binding mode that facilitates large HER2 cross-linking on the cell surface, significantly enhancing internalization and subsequent lysosomal localization[142]. This significantly promotes the internalization as well as subsequent lysosomal localization[143,144]. These findings underscore the suitability of bispecific antibodies for BsADC design. Subsequently, three mutations—L234F, S239C, and S442C—were introduced to the Fc region. Two engineered cysteine residues per heavy chain (S239C and S442C) enabled site-specific conjugation of the tubulysin warhead, a microtubule polymerization inhibitor, to the antibody *via* a maleimidocaproyl linker. This site-specific conjugation strategy ensures the formation of homogeneous BsADCs (DAR = 4). The introduction of the L234F mutation effectively decreases FcγR binding, leading to reduced non-specific uptake and off-target toxicity. Preclinical data on MEDI4276 demonstrates its effectiveness in inducing cell death in cell lines resistant

to T-DM1, highlighting a bystander killing effect. Additionally, MEDI4276 holds potential for use in patient groups that do not meet the conditions for current HER2-targeted therapy, offering a promising avenue with acceptable safety. However, the phase I data indicates that MEDI4276 has relatively serious side effects and poor PK properties[145]. In comparison to the safety profile of ZW49, the lower maximum tolerated dose (MTD) of MEDI4276 could be influenced by its valence, payload, and antibody configuration, signaling the need for further optimizations. While improvements in internalization show promise at the experimental level, the translation of theoretical drug design strategies into clinical benefits warrants further research. In summary, the unique advantages of biparatopic HER2-targeting BsADCs, with their ability to complementarily target different epitopes of HER2, induce antibody−receptor clustering on the cell surface. This increased internalization can effectively degrade and down-regulate the receptor, contributing to safety and efficacy while overcoming potential drug resistance. Further exploration of the clinical benefits holds promise.

3.1.2. Dual-antigen BsADC targeting HER2

Except for the resistance of internalization, internalized HER2−ADC complex could be recycled to plasma membrane while escaping lysosome degradation. Optimizing the internalization and lysosomal transport of HER2-targeting ADCs holds the potential to enhance cytotoxicity and reduce the HER2 threshold for clinical application. Approved HER2 ADC T-DM1 benefits approximately half of HER2-positive patients (HER2 over-expression defined as IHC3+ or FISH amplification ratio ≥ 2)[146]. Even in cases of high HER2 expression ($>10^6$), only a small portion undergoes internalization and degradation for payload release. The optimization of internalization and lysosomal transport could improve the therapeutic efficacy of HER2-targeting ADCs. In this context, dual-target BsADCs, which simultaneously target HER2 and fast-turnover receptors (such as CD63 and PRLR), offer a promising strategy to significantly improve intracellular drug delivery. This approach could potentially broaden the clinical applicability of HER2-targeting therapies and increase their effectiveness across a wider range of patients with varying HER2 expression levels.

BsADC on HER2×CD63. CD63, a member of the tetraspanin superfamily, exhibits widespread but not ubiquitous expression. It is primarily localized on the cell surface, late endosomes, and lysosomes[147]. The cytoplasmic domain of CD63 contains a YXXØ consensus motif, enabling its interaction with Aps and the recruitment of a clathrin coat[44,148,149]. This interaction with AP2/AP3 and clathrin positions CD63 as an adaptor protein, facilitating the extended trafficking of associated proteins from the cell surface to the late endosome−lysosomal compartment[44,45]. The presence of CD63 in these cellular compartments makes it a potential target for BsADCs aiming to enhance internalization and lysosomal transport, ultimately improving drug delivery and therapeutic efficacy.

The approach of introducing histidine substitutions in the variable heavy and light chain domains of the CD63-specific monoclonal antibody 2192 is a strategic move to modulate the affinity to CD63, thus ensuring tumor specificity[150]. By combining this low-affinity mutant arm with another Fab arm from the HER2 antibody 153, a BsAb targeting HER2 × CD63 is created. This design leverages antibody-dependent receptor crosslinking to enhance the effective internalization of HER2 and promote lysosomal co-localization[151]. Subsequently, BsHer2 × CD63 was

conjugated to anti-mitotic agent duostatin-3 *via* the VC linker[25,150]. However, the observed lack of efficacy in low-HER2 tumors indicates the necessity for further optimization, including potential enhancements in DAR and addressing tumor heterogeneity.

BsADC on HER2×PRLR. Prolactin receptor (PRLR), as an overexpressing target in malignant breast epithelium, could effectively mediate clathrin-dependent initial internalization and lysosome transportation through auto-ubiquitination and stimulating the recruitment of the AP2 complex. Low PRLR expression level ($10^4 - 2 \times 10^4$) is enough to effectively internalize the PRLR-targeted ADC[152]. To solve the unmet clinical needs of patients with low/medium HER2expression, BsADCs on PRLR could non-covalently cross-link HER2 and PRLR on the plasma membrane and trigger HER2 degradation in lysosomes.

By using the "knobs-into-holes" approach, the BsAb with HER2 arm and PRLR arm was designed[153]. The BsADC was conjugated to DM1 (a maytansine derivative payload) *via* surface lysine conjugation and non-cleavable linker (succinimidyl *trans*-4-[maleimidylmethyl] cyclohexane-1-carboxylate, SMCC), with the average DAR of 3.3[24]. Compared with the highly expressed HER2 ($\sim 10^6$), the lower expression ($\sim 10^4$) PRLR on the cell surface is sufficient to lead the constitutive internalization and subsequent lysosomal degradation. This indicates that high turnover surface targets could mediate good internalization and lysosomal degradation to improve the efficacy of BsADCs, even with low expression.

BsADC on HER2×APLP2. The tyrosine contained in the cytoplasmic tail of APLP2 contains overlapping tyrosine-based NPXY and YXXØ motifs. Similarly, APLP2 could bind to AP-2, mediate effective internalization and direct to lysosomal degradation after clathrin-mediated endocytosis[154]. APLP2 could deliver MHCI complexes and antigen−antibody complexes into the lysosomal degradation pathway[154,155] *via* full-length bispecific antibody technology[156], the BsAb with one αHER2 (Herceptin) arm and one arm of a αAPLP2 antibody was designed, which can bound to recombinant ECD of both APLP2 and HER2 simultaneously[157]. Through the site-specific coupling strategy of glutamine transaminase to ensure stability, AmPEG6-MMAD was conjugated on the BsAb with DAR = 2 or 4[157]. APLP2 targeting could effectively redirect recycled targets to lysosome to release payloads.

A recent abstract in AACR also reported that BsADC targeting HER2 × TROP2 shows higher internalization and drug effects in tumor cells co-expressing HER2/TROP2[158]. These BsADCs have proven that targeting rapid constitutive turnover targets could quickly accumulate and release payloads in lysosome, relying on the co-expression. The dual-target binding strategy also allows other poorly internalized targets to design future BsADCs, broadening the possible target selection.

HER2-targeting ADCs have revolutionized the chemotherapy strategies of breast cancer, especially T-DM1 and DS-8201[159]. Current HER-2 targeting ADCs still face unsolved challenges: (1) The demand for high expression levels of HER2 hinders further widespread application[160]; (2) It is necessary to further elaborate and address the toxicity status, response and drug resistance[161]; (3) Instead of single targeting, broadening the pipeline beyond HER2 is promising. Effectively internalized BsADCs are effective in cancer with low expression of HER2 or ERBB2 mutations beyond traditional HER2-positive breast cancer[153]. Besides, unique bispecific targeting could broaden the range of optional targets (*e.g.*, Trop-2[158,162]) for ADC design (Fig. 5).
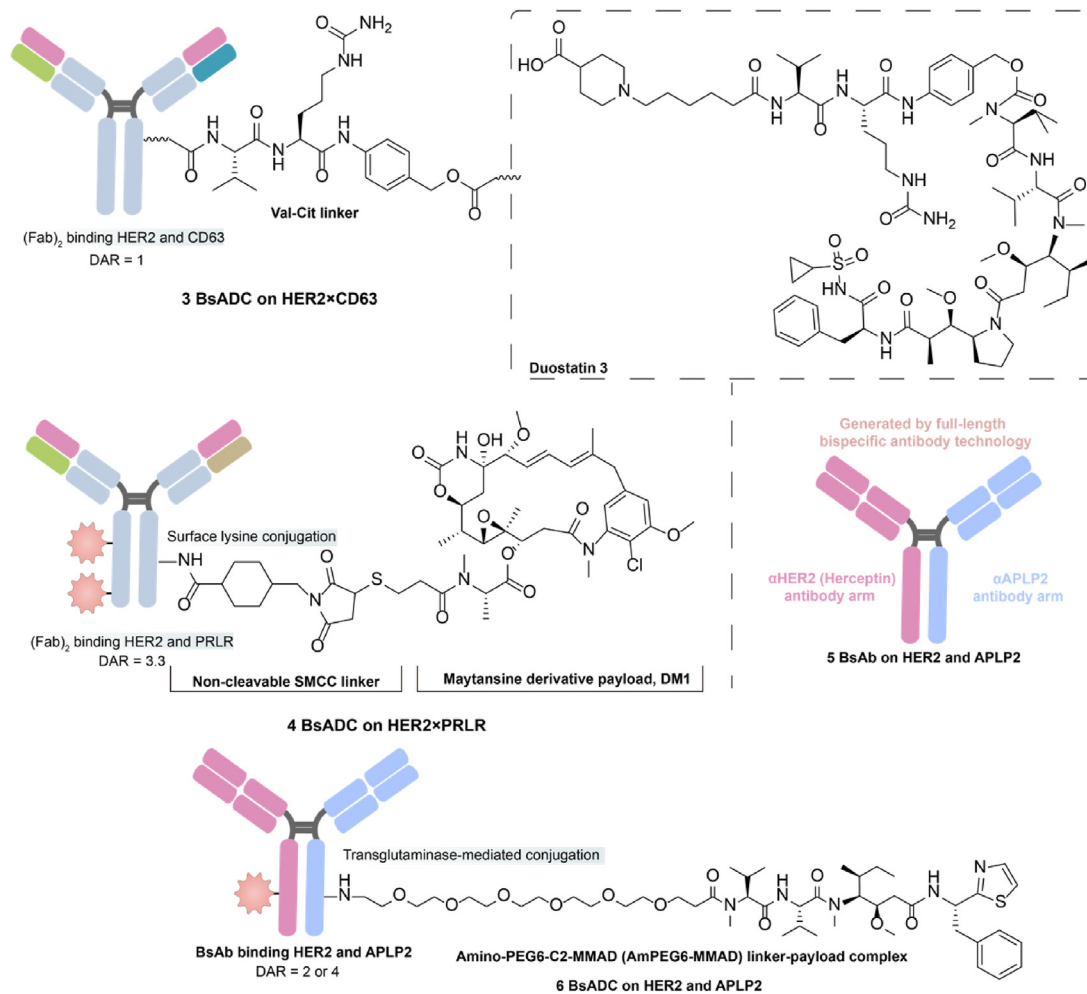
Figure 5 The design of dual-antigen BsADC on HER2.

3.2. BsADCs on EGFR and MET

EGFR, a member of the ERBB receptor tyrosine kinase family, plays a pivotal role in regulating essential functions within epithelial malignancies, thereby inducing disturbances in homeostasis[163]. In clinical applications, TKIs and mAbs are commonly employed to impede EGFR activity. Notably, anti-EGFR mAbs such as cetuximab and panitumumab have undergone clinical evaluations across various EGFR-amplified cancers. However, the emergence of clinical drug resistance has resulted in a median non-progressive survival period of less than 12 months[164]. The primary contributors to resistance during anti-EGFR antibody treatment are acquired genomic alterations (Acq-Gas) induced by treatment pressure. Specifically, mutations in RAS, BRAF, or the EGFR-ectodomain, as well as amplifications of ERBB2 (HER2) or MET, have become predominant factors[164,165]. Depatuxizumab mafodotin, an ADC targeting EGFR, was developed for the treatment of glioblastoma. However, clinical data failed to demonstrate a significant improvement in overall survival[166]. Clinical trials have explored the potential of combined MET inhibitors in patients with EGFR mutations and MET disorders as a strategy to overcome drug resistance[167−169]. BsADCs show promise in addressing anti-EGFR resistance

mechanisms, including sensitizing mutations and the activation of bypass pathways[170,171].

3.2.1. Dual-epitope BsADC on EGFR

Patients undergoing clinical administration of Cetuximab or Panizumab frequently manifest Acq-Gas, such as S492R, G465R, G465E, and I491M, resulting in the abrogation of interaction and consequent resistance to ADCs targeting EGFR[164]. To mitigate the emergence of drug resistance, a biparatopic antibody was developed, targeting two distinct epitopes. This biparatopic antibody was engineered by fusing nanoantibodies specific to non-overlapping epitopes (9G8 and 7D12) on EGFR[172]. MMAE was conjugated on the BsAb by engineered surface cysteine S7C on 7D12 part of 97m conjugate, and the tetravalent biparatopic ADC was demonstrated with homogeneous DAR = 1.83[53]. Subsequently, an E430G mutation was introduced into the Fc region to enhance complement-mediated immune responses. Functioning as a tetravalent biparatopic BsADC, 7D12 disrupted the EGFR signal cascade, while 9G8 stabilized the tethered conformation of EGFR ECD, sterically preventing dimerization. Additionally, 7D12 and 9G8 exhibited efficacy against different EGFR mutant cell lines, inducing a more potent CDC effect on NIH-3T3 cells expressing wild-type EGFR or exhibiting cetuximab resistance mutations.

The tetravalent biparatopic BsADC demonstrated its ability to overcome the limited combination interface associated with cetuximab resistance mutations, exhibiting enhanced internalization, conjugation efficiency, and therapeutic efficacy. The utilization of a dual epitope binding mode in BsAbs for designing multivalent ADCs presents a promising strategy to circumvent clinical drug-resistant mutations.

3.2.2. Dual-antigen BsADCs on EGFR

BsADC on EGFR×HER3. Resistance to EGFR-targeted therapy may also occur through compensatory signaling *via* the ERBB receptor, involving receptors such as HER2 and HER3, suggesting the potential for combination therapy[173,174]. Nevertheless, clinical data indicates that the combination of patuizumab and cetuximab results in overlapping toxicity, rendering the combination intolerable. HER3 is notably overexpressed in EGFR-resistant cancer cell lines, contributing to both internal and external resistance to EGFR while activating PI3K[175,176]. Recognizing HER3 as a novel target to overcome EGFR resistance, therapeutic combinations targeting both EGFR and HER3 are under evaluation, including BsADCs[177,178]. A BsAb named SI-B001, targeting both EGFR and HER3, was constructed by utilizing the anti-EGFR Fab and anti-HER3 scFv[179]. The Topoisomerase I inhibitor (TOPIi) ED04 was conjugated to cysteine sites on SI-B001 *via* a novel cleavable AC linker, resulting in the design of a novel BsADC named BL-B01D1 with a DAR of approximately 8[180].

SI-B001 exhibits specificity in targeting both EGFR and HER3, enhancing safety. BL-B01D1, in turn, facilitates synergistic internalization through the crosslinking of its dual targets[179]. By combining SI-B001 with small molecule toxins, BL-B01D1 achieves targeted killing of EGFR-dependent tumors, mitigating drug resistance arising from HER3. In a recent conference, the Phase I trial in patients with solid tumors (NCT05194982) revealed promising efficacy and a favorable safety profile[181]. Particularly in patients with EGFR-mutant NSCLC, the ORR reached 63.2%, demonstrating therapeutic efficacy against various drug-resistant mutations. Additionally, the ORR in EGFR-wild NSCLC reached 44.9%. The most common TRAEs (>10%, all grade/$\geq$ G3) were leukopenia (60%/30%), neutropenia (51%/34%), anemia (45%/15%), thrombocytopenia (44%/19%), alopecia (30%/0%), nausea (29%/<1%), vomiting (28%/0%), asthenia (21%/<1%), decreased appetite (22%/<1%), asthenia (21%/<1%), hypophagia (16%/0%), diarrhoea (15%/2%), mouth ulceration (15%/<1%), and rash (13%/0%), with no reported cases of interstitial lung disease[181]. In November 2023, BL-B01D1 has become the first BsADC entering phase III trial (NCT06118333). Despite these encouraging results, further safety evaluation and optimization based on early data remain imperative.

BsADC on EGFR×c-MET. Several BsAbs targeting both c-MET and EGFR have been reported, demonstrating a synergistic effect in inhibiting tumor proliferation and metastasis[182−184]. In the design of BsADCs, careful selection of appropriate epitope combinations is crucial to avoid complete or partial agitation of c-MET[185]. Given the widespread expression of targets throughout the body, it becomes imperative to target tumor-specific epitopes and optimize dual-arm affinity to enhance tumor selectivity[186].

Through the screening of EGFR and c-MET antibodies with varying affinities and epitopes, it has been observed that lower affinity in the EGFR arm correlates with higher tumor selectivity and improved internalization effects[74]. The specific mechanism underlying enhanced internalization remains unclear but may be associated with the cross-linking of c-MET and EGFR, along with

the co-internalization effect of the dual-target binding mode. Antibodies occupying overlapping epitopes with B10 and B10v5 on c-MET may also contribute to promoting internalization. Utilizing the strand exchange engineered domain (SEED) technology[187,188], a c-MET × EGFR BsAb was constructed by incorporating anti-c-MET Fab and anti-EGFR scFv, while avoiding light chain mispairing[74]. Given the favorable tumor selectivity and appropriate internalization effects exhibited by the generated BsAb, MMAE was employed for C-terminal sortase-mediated conjugation on both heavy chains *via* a protease-cleavable VC linker, resulting in the DAR of 2[74]. The abstract of AZD9592, a BsADC targeting c-MET × EGFR, has recently been reported[189]. The BsAb was generated on the DuetMab platform, which was redesigned by introducing a Fab disulfide bond to prevent light chain mispairing. Adjusting the affinity relationship between c-MET and EGFR (c-MET: EGFR = 15) has been shown to reduce the side effects associated with EGFR targeting. The TopIi AZ14170132 was conjugated *via* a cleavable linker to design the BsADC, exhibiting good selectivity, efficacy, and safety in *vitro* studies. Currently, AZD9592 is undergoing evaluation in a phase I clinical trial. For BsADCs targeting EGFR × c-MET, a critical consideration is how *in vitro* selectivity can be translated into *in vivo* safety. This transformation necessitates the utilization of a suitable model for further stability and pharmacokinetic research, addressing the important question of ensuring efficacy and safety in a real-life, physiological context.

3.2.3. Dual-epitope BsADCs on MET

The Hepatocyte Growth Factor (HGF)-Mesenchymal-Epithelial Transition Factor (MET) pathway plays a significant role in cancer development across various tumor types and stages, from initiation to metastasis[190]. The up-regulation and amplification of MET are considered major escape routes during anti-EGFR therapy[76,191−193]. C-MET can cross-react with EGFR, conferring resistance to EGFR-targeted therapy, making inhibition of c-MET a viable strategy to overcome EGFR resistance[190]. The recent success of MET-directed TKIs in treating NSCLC harboring MET exon 14 mutations has elevated MET as an attractive therapeutic target[194]. MET-targeting therapy, especially selective MET TKI[194] and ADCs[195], is progressing.

Despite progress, clinical results of MET inhibitors face challenges[196]. Recent Phase II clinical data of the anti-c-MET ADC Teliso-V showed limited activity in patients with EGFR mutations overexpressing MET[197,198]. Current clinical dilemmas of MET-targeted ADCs include: (1) Lack of consensus on the optimal diagnostic cut-off point for MET copy number[199]; (2) Secondary mutations in the MET kinase domain or alterations in bypass signals promoting resistance[200]; (3) The need for further optimizations in internalization, transportation, and degradation efficiency[201]. These challenges highlight the complexity in developing effective MET-targeted therapies and the importance of addressing various factors to enhance their clinical utility.

In contrast to conventional MET-targeting ADCs, the design of a biparatopic MET × MET BsADC offers innovative solutions to overcome existing challenges. The MET biparatopic antibody has the capability to form a 2:2 antigen−antibody complex, facilitating effective MET internalization and lysosomal transport, rendering it an ideal candidate for ADC design[201,202]. By conjugating the Maytansinoid payload M114 *via* a protease-cleavable linker to the surface lysine of the MET BsAb, REGN5093-M114 was characterized with the average DAR of 3.1[202]. The
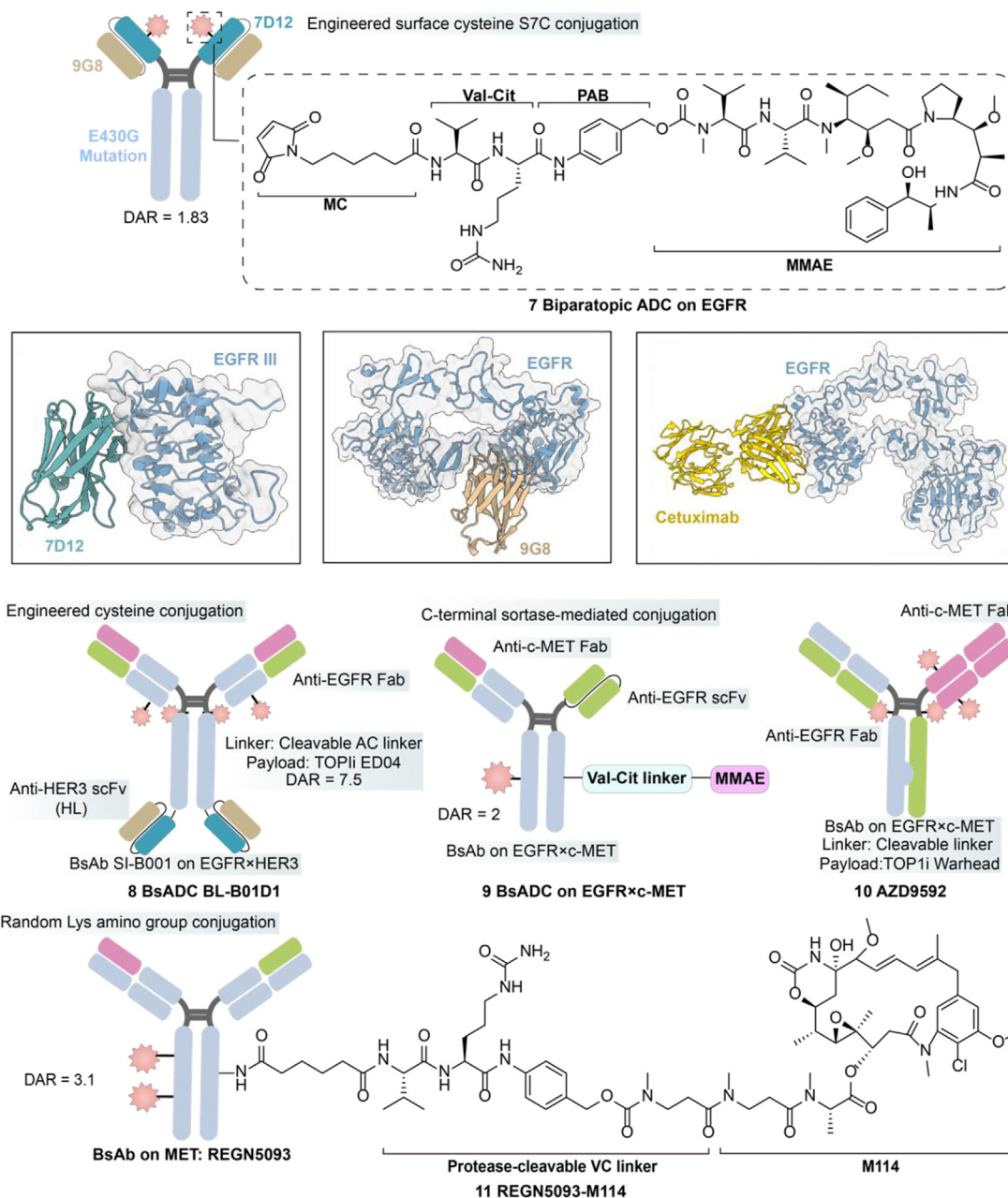
Figure 6 BsADC on EGFR and MET; Interactions of 7D12 (blue PBD id:4KRL), 9G8 (brown PBD id: 4KRP) and Cetuximab (yellow PBD id: 1YY9) with EGFR ectodomain.

cleavable VC linker is advantageous for releasing payloads in the late and recycling endosomes with a weakly acidic environment[43].

Considering the expression of MET in normal tissues, payloads lacking bystander killing may exhibit lower side effects. Preclinical data indicates that REGN5093-M114 significantly inhibits the proliferation of MET-overexpressed NSCLC cells[198]. It holds promise for evaluating the surface expression levels of MET as a criterion for accepting MET-targeting therapy. A phase I, dose-escalation, and dose-expansion study has been initiated to evaluate REGN5093-M114 in adult patients with MET-overexpressing advanced cancer (NCT04982224)[203]. REGN5093-M114 is anticipated to exert therapeutic effects in MET amplification and EGFR-resistant patients, although the tolerance of combined

application with EGFR-targeted drugs requires further evaluation[168].

In addressing challenges beyond Acq-Gas, antibody-targeted therapies for EGFR and MET must contend with additional complexities. Given the ubiquitous expression of EGFR, it becomes imperative to design EGFR antibodies with varying affinities to mitigate systemic toxic side effects[204]. In cases where the oncogenic stimulus arises downstream, particularly in tumors with KRAS mutations, agents blocking EGFR prove ineffective[205]. A current strategy involves enhancing the function of the Fc region of EGFR antibodies, with a specific focus on ADCC[206,207]. Furthermore, clinically observed mutations in EGFR, such as exon 20 insertions, present novel challenges. Identifying and
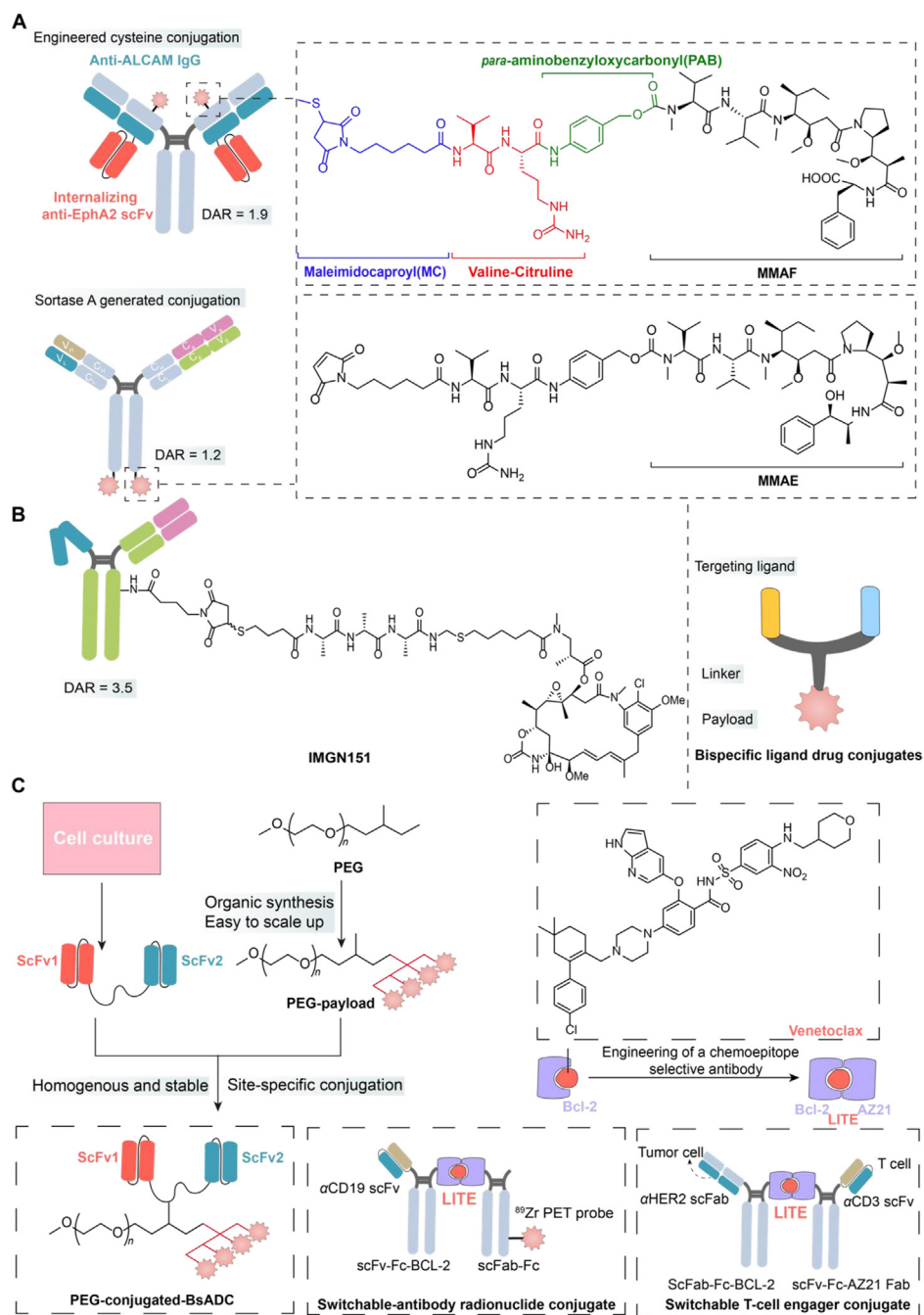
Figure 7 Novel BsADCs beyond traditional paradigm. (A) BsADCs beyond traditional target paradigm, targeting EphA2 and pMHC; (B) Novel FRα targeting BsADC, IMGN151, and schematic diagram of bispecific ligand drug conjugates; (C) Novel BsADCs beyond traditional antibody paradigm, including PEG-conjugated BsADC and LITE platform.

developing novel therapies targeting these rare driver mutations holds clinical significance[208].

The distinctive SEMA domain of MET facilitates easier dimerization than EGFR upon activation by HGF[209]. Subsequently, the activated MET dimer undergoes autophosphorylation at tyrosine residues in the intracellular kinase domain and the substrate docking site[210,211]. The potential promotion of dimerization by the divalent structure of MET antibodies may contribute to a suboptimal therapeutic effect. In the future design of biparatopic MET × MET BsADCs, careful consideration of

appropriate epitope selection and antibody construction strategies is essential to avoid undesired dimerization and autophosphorylation[212]. When designing BsADCs targeting MET × EGFR, it is crucial to prevent the phosphorylation of both EGFR and MET. This involves adjusting the affinity ratio of both arms while ensuring optimal binding efficacy. Additionally, determining the clinically applicable population for BsADCs requires detailed discussions on the expression levels of MET and EGFR in clinical tumors. This consideration is essential for optimizing the therapeutic potential of these innovative therapeutic modalities (Fig. 6).

3.3. Novel BsADCs beyond traditional paradigm

BsADCs have remarkably expanded the scope of potential targets and scaffolds beyond the conventional paradigm. While most ADCs currently target tumor surface antigens, their expression in normal tissues can lead to off-target toxicity. Antibodies targeting major histocompatibility complex proteins (pMHC), known as TCR-mimic (TCRm) antibodies, offer a promising solution. TCRm antibodies can identify tumor antigens expressed at low levels on tumor cells while being absent in normal tissues[213,214]. Several reported TCRm BsAbs exhibit anti-tumor potential with significantly reduced off-target toxicity[215,216]. However, the low epitope density of pMHC (tens to thousands) results in suboptimal effectiveness for TCRm antibodies and ADCs.

To enhance the efficacy of TCRm-ADC, bispecific antibody strategies are employed. WT1 and ny-eso-1 antigens, overexpressed in solid tumors with overlapping expression profiles and strong immunogenicity, were targeted with TCRm antibodies. Using "knobs-into-holes" technology, two Fab arms of TCRm antibodies were combined. Subsequently, Bi-TCRm-ADC was designed through sortase A-generated site-specific conjugation to MMAE. Bi-TCRm-ADC demonstrated significantly improved effectiveness compared to TCRm-ADC (IC_{50} = 50 nmol/L, DAR = 1.2)[217]. TCRm is not targeted to normal tissues, leading to a substantial reduction in off-target toxicity. Compared with TCRm-ADC, Bi-TCRm-ADC is expected to address the low drug effects caused by the generally low expression of pMHC. However, the challenge of identifying two simultaneous high-expression pMHCs on tumors remains a key consideration for future development.

Indeed, BsADCs offer an effective means to facilitate the conversion of non-internalized antigens into internalized antigens. For example, EphA2, characterized by rapid internalization, and activated leukocyte cell adhesion molecule (ALCAM), a non- or slowly internalizing antigen, were utilized to generate a BsAb[218]. Intriguingly, when the ratio of EphA2 to ALCAM on the cell surface surpassed the threshold of 0.2, bispecific antibodies demonstrated efficient internalization. Conversely, when the ratio fell below this threshold, internalization was impeded. The MC-VC-pab-MMAF payload complex was site-specifically conjugated via cysteine residues. In the context of bispecific binding by BsADCs, the internalization effect can be significantly influenced by simultaneously targeting neighboring antigens, contingent upon their expression ratio. This capability broadens the spectrum of antigen selection to include poorly internalized types for the purpose of designing BsADCs. This innovative approach provides a nuanced strategy for enhancing the internalization potential of certain antigens, contributing to the versatility and efficacy of BsADCs in targeting a wider range of tumor antigens.

In 2022, the FDA granted accelerated approval to the novel ADC IMGN853 (ImmunoGen™), targeting folate receptor alpha (FRα)[219]. To enhance the therapeutic efficacy in patients with medium/low FRα levels, a biparatopic ADC, IMGN151, was designed[220,221]. IMGN151 retained the Fab of IMGN853, and a scFv based on the VL-Linker-VH-Fc format was employed to construct the BsAb. To prevent aggregate formation of scFv, molecular optimizations, including VH44-VL100 cysteine mutation, were implemented. Additionally, modification of C220S in Fc aimed to reduce free Cys. The Maytansinoid derivative DM21 was linked to the BsAb via a stable cleavable peptide linker. The DM21-L-G linker−payload complex contributed to its membrane penetration and pharmacokinetic characteristics. IMGN151 exhibited up to 200 times greater activity against four FRα-medium cell lines. Moreover, IMGN151 induced complete tumor regressions across high/medium/low FRα expression levels with good toleration, and it is currently undergoing a phase I trial. Beyond ADCs, bispecific ligand drug conjugates targeting FRα also show promise, with CBP-1018 and CBP-1008 being pioneers in clinical trials[222−224]. These advancements represent significant strides in the development of targeted therapies, offering new avenues for treating cancer with diverse FRα expression profiles (Fig. 7B).

The introduction of various new technologies has revolutionized the generation of BsADCs, transcending the traditional ADC paradigm and presenting distinct advantages. One notable advancement is the PEG-conjugated-BsADC (P-BsADC), which not only ensures uniform coupling production but, due to its small molecular weight and absence of Fc fragments, exhibits high endocytosis efficiency, tissue penetration, and reduced toxicity. Another noteworthy approach involves the use of a bispecific peptide-polymer created by 8-arm PEG, simultaneously conjugating PD1-binding and PDL1-binding peptides[225]. Furthermore, the ligand-induced transient engagement (LITE) of multiple antibody domains is a cutting-edge technique that combines the prolonged half-life advantages of biologic drugs with the precise temporal control of activity associated with small molecules[226]. These innovative strategies hold great promise in achieving therapeutic efficacy while minimizing off-target effects, representing a significant step forward in the evolution of BsADC technologies.

4. Conclusions and perspectives

BsADCs, embodying a "1 + 1" model, represent a novel therapeutic class that amalgamates the strengths of ADCs and BsAbs. This fusion leverages the anti-tumor mechanisms of ADCs, while addressing clinical challenges traditionally associated with ADCs through the versatility of BsAbs. BsADCs present a promising avenue for significantly enhancing the therapeutic efficacy of traditional ADCs, transcending conventional target and scaffold paradigms. Recent advancements have propelled BsADCs into phase I/II clinical trials, marking substantial progress in their development. Nevertheless, challenges persist, primarily attributed to the complexity of solid tumors, encompassing factors, compartmental heterogeneity, histological disorder, and poor penetration. It is imperative to refine the design strategy of BsADCs to overcome these challenges. In this review, we encapsulate the latest design considerations and advances in BsADCs, with the goal of charting a course for future drug design. While existing progress is noteworthy, the continued development and application of BsADCs face unique challenges in the following aspects.

4.1. Broadening the antibody skeleton

The current target selection for BsADCs remains somewhat limited, primarily focusing on HER2, c-MET, and EGFR. However, bispecific strategies have the potential to broaden the range of targets to include those with poor internalization or low expression. Considering the diverse types of BsAbs, further enriching the antigen selection for BsADCs could diversify the anti-tumor mechanisms. Promising avenues include immune-modulating BsADCs based on T-cell engaging or PD-L1 targeting BsAbs. The reason of limited antibody skeleton might be the difficulty of finding suitable co-expressed antigen

combinations. The limited antibody skeleton options may stem from the challenge of finding suitable co-expressed antigen combinations. Targeting specifically expressed or highly internalized antigens with one arm of BsAbs can enhance the overall drug effect[218]. The design of non-internalized BsADCs, utilizing moderately stable cleavable linkers and bystander-effect payloads, holds promise by expanding optional targets to the extracellular compartment[95]. Beyond IgG-like BsAbs, non-IgG-like BsAbs (lacking an Fc region) offer advantages such as a simple structure, good permeability, and low immunogenicity. Addressing their half-life challenges through long-term platform technologies like polyethylene glycol modification and Fc fragment fusion is a worthwhile avenue for further exploration. As a specificity beyond BsADCs, multispecific antibodies, especially trispecific[227] and four-in-one[228,229] antibodies, represent a new opportunity. These are expected to address challenges such as receptor redundancy, off-target toxicity, and the heterogeneity of solid tumors. This approach also has the potential to cater to more personalized and precise treatment needs in the future.

4.2.   Clearing the heterogeneous coupling orientations

Constructing BsADCs through random chemical coupling of payloads based on functional groups carries the risk of heterogenic coupling orientations[230]. This heterogeneity could compromise the bispecific binding mode and alter the physical and PK properties of BsADCs[231]. Site-specific conjugation strategies could generate BsADCs with homogeneous DAR. In the context of random coupling, evaluating the potential impact of conjugation strategies on antibody binding patterns is crucial[230]. This meticulous assessment is expected to contribute to the improvement of side effects and poor drug effects caused by uneven DAR in BsADCs constructed through random coupling. Homogeneous DAR not only enhances the precision of drug delivery but also ensures the consistency of the therapeutic response, making it a critical consideration in the design and development of BsADCs. It's crucial to highlight that the foundation of site-specific conjugation lies in the careful selection of the conjugation sites, a pivotal factor influencing the drug efficacy of ADCs.

4.3.   Uniforming bispecific antibody generation

The structural incongruity between heavy and light chains presents challenges in chain association during the construction of BsAbs using hybridoma technology, resulting in compromised yield and purification efficiency[71]. The advent of modern genetic engineering technologies, particularly the "knobs-into-holes" strategy, has yielded significant breakthroughs in overcoming this challenge. Subsequently, protein engineering has emerged as a comprehensive approach for BsAb generation, albeit with inherent complexity and elevated costs. The intricate production process has, in turn, led to extensive screening characterized by low yield, variable quality, and non-negligible impurities. In contrast to protein engineering, chemical modification of antibodies has evolved as a novel, modular, and reproducible production strategy. This chemical strategy demonstrates favorable yield, enabling the rapid generation of stable, homogeneous, and precisely defined BsAbs. Furthermore, the exploration of innovative BsAb generation platforms holds promising prospects[232].

4.4.   Clarifying the parameters between two targets

The multivalent binding mode and parameters of BsADCs impose mutual restrictions and dependencies. Design considerations between two targets warrant meticulous examination, particularly with respect to variations in affinity magnitudes, expressions, and valence. The bispecific binding mode not only influences one another *via* valence but also impacts overall internalization through cross-arm binding[233]. The antibody structure and the molecular distance between binding epitopes further contribute to binding dynamics, influencing the characteristics of the antigen−antibody complex and subsequent therapeutic activity[142]. Beyond affinity, the expression threshold of surface antigens on cancer cell lines plays a pivotal role in determining overall drug effects[218]. Addressing these critical points necessitates comprehensive screening of combinations of binding arms with varying affinities to achieve optimal biological activity. Despite the substantial workload, the integration of AI and machine learning technologies, such as the RF diffusion model, is anticipated to confer a newfound advantage in both generating and evaluating BsAbs based on diverse binding arms[234].

4.5.   Piggybacking and hijacking the transportation

BsAbs can navigate through approaches that leverage the initial specificity of a BsAb solely as a transport modality for the second specificity, a phenomenon inherently obligate and reliant on sequential binding, referred to as "piggyback" or "hijacking"[64]. An illustrative example is the BsAb platform utilizing low-affinity transferrin receptors to piggyback a *β*-secretase antibody, facilitating effective passage through the blood−brain barrier[48,235,236]. We have reported that BsADCs targeting CD63, PRLR, and APLP2 demonstrated the ability to hijack lysosomal trafficking. Furthermore, the targeting of EphA2 not only facilitates BsADC passage through the blood−brain barrier but also enhances drug internalization, underscoring its efficacy as a preferred target for BsADCs. Building upon these strategies, there is promise in designing BsADCs that adeptly traverse biological barriers and evade lysosomal degradation.

4.6.   Addressing potential safety issues

While BsADCs are dedicated to enhancing specificity and thereby mitigating off-target toxicity and side effects, early clinical data, exemplified by ZW49, MEDI4276, and BL-B01D1, indicates a less promising clinical safety profile than anticipated. Off-target toxicity emerges as a predominant characteristic in ADC-related toxicity. However, relying solely on BsAbs may not sufficiently address the imperative to reduce off-target toxicity. In addition to antibody innovation, crucial discussions surrounding the linker−payload complex are indispensable, encompassing considerations of linker stability, a homogeneous conjugation strategy, DAR, and bystander killing effect. Given the multifaceted reasons for potential toxicity associated with BsADCs, it becomes imperative to optimize the antibody, linker, and payload components synergistically[8]. Beyond the payload−linker complex, strategies for dose optimization and dosing methods, such as subcutaneous administration[237], hold promise in addressing the dose-dependent toxicity of BsADCs and improving the therapeutic index[238]. The incorporation of pharmacogenomics analysis in early clinical trials is a promising avenue to discern the characteristics of patient

populations at a higher risk of adverse events following BsADC treatment[239].

In summary, the advent of unique bispecific targeting modes has infused fresh innovation into the field of ADCs, marking a new generation of ADCs. Despite being in the early stages of development, BsADC presents a novel approach with considerable potential. However, early clinical trials encounter substantial challenges. Anticipations are high that the implementation of the aforementioned strategies will lead to the successful design of innovative BsADCs. This achievement would hold significant importance in surmounting the existing clinical challenges faced by traditional ADCs and in the creation of more precisely targeted drugs. Beyond BsADCs, a series of next-generation strategies promises epoch-making contributions to novel ADC design. These include ADCs featuring dual payloads[240], immune-modulating ADCs[86], radionuclide drug conjugates[241], masked ADCs[242], ADC combination therapy[243], and peptide−drug conjugates[244]. The future entails evaluating the application of these strategies, either individually or in combination, for the design of novel ADCs, presenting significant prospects for advancing the field.

Acknowledgments

Author contributions

Yuxi Wang contributed to the idea and gave valuable suggestions; Yilin Gu drafted the manuscript and painted the figures; Zhijia Wang revised the manuscript. All authors have approved the final review and the submission.

Conflicts of interest

The author declare that no conflicts of any interests exist.

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