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New Technologies Bloom Together for Bettering Cancer Drug Conjugates

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Abstract——**Drug conjugates, including antibody-drug conjugates, are a step toward realizing Paul Ehrlich's idea from over 100 years ago of a "magic bullet" for cancer treatment. Through balancing selective targeting molecules with highly potent payloads, drug conjugates can target specific tumor microenvironments and kill tumor cells. A drug conjugate consists of three parts: a targeting agent, a linker, and a payload. In some conjugates, monoclonal antibodies act as the targeting agent, but new strategies for targeting include antibody derivatives, peptides, and even small molecules. Linkers are responsible for connecting the payload to the targeting agent. Payloads impact vital cellular processes to kill tumor cells. At present, there are 12 antibody-drug conjugates on the market for different types of cancers. Research on drug conjugates is increasing year by year to solve problems encountered in conjugate design, such as tumor heterogene-**ity, poor circulation, low drug loading, low tumor uptake, and heterogenous expression of target antigens. This review highlights some important preclinical research on drug conjugates in recent years. We focus on three significant areas: improvement of antibody-drug conjugates, identification of new conjugate targets, and development of new types of drug conjugates, including nanotechnology. We close by highlighting the critical barriers to clinical translation and the open questions going forward.**

Significance Statement——**The development of anti-cancer drug conjugates is now focused in three broad areas: improvements to existing antibody drug conjugates, identification of new targets, and development of new conjugate forms. This article focuses on the exciting preclinical studies in these three areas and advances in the technology that improves preclinical development.**

I. Introduction

Several drug conjugates for cancer therapy have gained clinical use. The pace of clinical advancement appears to have quickened in recent years, drawing more attention to their preclinical development. Drug conjugates are a category of antitumor therapeutic agents with promising prospects for clinical efficacy (Theocharopoulos et al., 2020). A typical drug conjugate is formed by chemically attaching a payload (or small molecule toxin) to a targeting agent (such as an antibody) via a linker. The targeting agent transports the payload specifically into tumor cells to inhibit tumor growth (Zhao et al., 2020). Clinically, the most common type of drug conjugates are antibody-drug conjugates (ADCs), of which there are now 12 with

approval from the US Food and Drug Administration (FDA) for different oncological indications (Table 1) (Hafeez et al., 2020). Gemtuzumab ozogamicin was the first FDA-approved ADC, which gained clinical use in 2000 for CD33$^+$ acute myeloid leukemia. The second ADC, brentuximab vedotin, was approved in 2011 to treat Hodgkin's lymphoma. From 2013 to 2018, three ADCs (trastuzumab emtansine, inotuzumab ozogamicin, and moxetumomab pasudotox) were approved (Hafeez et al., 2020), and from 2019 to 2021, seven additional ADCs arrived. The development of ADCs has clearly accelerated in recent years (Jin et al., 2021). At the same time, exciting preclinical research on ADCs and other drug conjugates is growing, with the hope of yielding other effective clinical agents (Manzano and Ocaña, 2020).

Although the development of drug conjugates is flourishing, the development of drug conjugates is arduous. As early as the beginning of the 20th century, Paul Ehrlich first proposed tumor-targeting agents that could carry chemical drugs specifically to a tumor site, leading to tumor cell destruction (Ehrlich, 1907, 1960; Strebhardt and Ullrich, 2008). The goal of ADC therapy is in line with this idea. However, although the concept was put forward very early, the ADC concept was first tested in the late 1950s with a methotrexate-linked antibody conjugate targeting leukemia (Mathe et al., 1958). Unfortunately, immunogenicity resulting from its animal origin and difficulties in its separation and purification led to its failure in clinical development. Due to the advent of monoclonal antibody technology in the 1970s, the purification and immunogenicity problems of ADCs were improved (de Chadarevian, 2011). The first modern ADC clinical trial was conducted in the 1980s, focusing on a carcinoembryonic antigen-targeted antibody-vindesine conjugate (Ford et al., 1983). Although this trial found no toxicity or hypersensitivity, this ADC did not ultimately gain approval for clinical use, due to lack of efficacy (Jabbour et al., 2021).

In the following decades, the resolution of some critical problems encountered in ADC development by advancing related technologies allowed the approval of the first-generation ADC (Mylotarg) (McGavin and Spencer, 2001). Such technological advancements include humanized antibody preparation, new target identification, ultra-toxin discovery, and precise methods for quality analysis (McGavin and Spencer, 2001). However, due to limited efficacy and fatally toxic events caused by early release of its payload, Pfizer withdrew Mylotarg from the market in 2010 (Norsworthy et al., 2018). The mentioned fatality was attributed to the early release of the cargo. Heterogeneity induced by a variable drug antibody ratio (DAR) and aggregation in circulation caused by high DAR additionally limited the effectiveness of Mylotarg in the clinic (Clarke and Marks, 2010). Significant efforts have focused on these aforementioned problems. During the decade after Mylotarg first got approval, research mainly focused on site-specific linker technology for homogeneous ADC preparation (Tolcher, 2016). Approaches like enzyme-based site-selective coupling reaction, chemical agent–based site-selective coupling reaction, and DAR determination methods were also investigated. In addition, essential concepts including internalization rate, tumor tissue penetrability, bystander effect, and payload-induced antibody conformational instability were gradually recognized (Tolcher, 2016). Based on this research, the second-generation ADCs, for example Adcetris and Kadcyla, were designed, prepared, evaluated, and approved (Fig. 1) (Garcia-Echeverria, 2014).

Currently, there are 12 ADC drugs marketed in the United States, and clinical research on many more ADCs is ongoing (Jin et al., 2021). More than that, several new preclinical studies on drug conjugates have emerged (Hafeez et al., 2020). This research has grown many new branches around solving problems with current conjugates and has brought about a variety of attractive new conjugate designs beyond the marketed ADC structures (Beck et al., 2017). Therefore, this review classifies the new preclinical development into three areas: (1) improvement to ADC components such as the antibody, linker, and payload; (2) identification of new targets for ADCs and other drug conjugates; and (3) evaluation of new targeting approaches (X-drug conjugates), where X can be small molecule, peptide, ligand, single-chain variable fragment (scFv), or nanoparticle. We hope this review will summarize the current state of the field and provide ideas for future research on drug conjugates.

II. New Technology in the Development of Antibody Drug Conjugates

A. Antibody Technology

The antibody portion of the ADC recognizes and specifically binds to the target tumor tissue, thus forming a targeted drug delivery system (Abdollahpour-Alitappeh et al., 2019). In recent years, there has been much research on ADC antibody technology, including optimization of cellular internalization rates and tumor permeability as well as improvements to the preparation of homogeneous ADCs to improve efficacy and reduce side effects.

1. Bispecific Antibody. Internalization is a standard process for most of the current ADCs, but few tumor antigens have ideal internalization characteristics. Figure 2 illustrates a selection of antibody configurations for ADCs, highlighting differences in internalization. One way to improve internalization is to pair a poorly internalized antigen with a rapidly internalized antigen. In this way, the rapidly internalized antigen will speed the uptake of the poorly internalized antigen. An example of this approach is found with a bispecific antibody targeting an activated leukocyte cell adhesion molecule. This bispecific antibody binds to both a noninternalizing tumor antigen (activated leukocyte cell adhesion molecule) and a rapidly internalizing antigen (ephrin receptor A2). When both are expressed on the surface of a leukemia cell, the bispecific antibody has an improved internalization rate and cytotoxicity both in vitro and in vivo compared with monospecific ADCs (Lee et al., 2019). A bispecific antibody was first described more than 50 years ago (Labrijn et al., 2019). Successive conceptual and technical innovations of bispecific antibodies yielded more than 100 known bispecific antibodies today, one-quarter of which have been commercialized or are in

TABLE 1
ADC drugs approved by FDA (up to March 2022)

	Brand Name	ADC Name	Indication	Target	Linker	Payload	Company	Launch Year
1	Mylotarg	Gemtuzumab ozogamicin (GO)	1. Newly-diagnosed CD33$^+$ acute myeloid leukemia 2. Relapsed or refractory CD33$^+$ acute myeloid leukemia	CD33	Cleavable Hydrozone linker	Calicheamicin	Pfizer	2000 Relaunched in 2017
2	Adcetris	Brentuximab vedotin (SGN-35)	Hodgkin lymphoma, large cell lymphoma, T-cell lymphomas	CD30	Cleavable Valine-citrulline linker	MMAE	Seattle Genetics/ Takeda	2011
3	Kadcyla	Trastuzumab emtansine (T-DM1)	HER2$^+$ breast cancer	HER2	Noncleavable Thioether linker	DM1	Roche	2013
4	Besponsa	Inotuzumab ozogamicin	Relapsed or refractory B-cell precursor acute lymphoblastic leukemia	CD22	Cleavable Hydrozone linker	Calicheamicin	Pfizer	2017
5	Lumoxiti	Moxetumomab pasudotox	Relapsed or refractory hairy cell leukemia	CD22	Fusion protein (antibody and payload)	Pseudomonas exotoxin	AstraZeneca	2018
6	Polivy	Polatuzumab vedotin	Relapsed or refractory diffuse large B-cell lymphoma	CD79b	Cleavable Valine-citrulline linker	MMAE	Roche	2019
7	Padcev	Enfortumab vedotin	Advanced or metastatic urothelial cancer	Nectin-4	Cleavable Maleimidocaproyl valine-citrulline linker	MMAE	Seattle Genetics/ Astellas	2019
8	Enhertu	Fam-trastuzumab deruxtecan (DS-8201)	1. Metastatic breast cancer 2. Locally advanced or metastatic gastric cancer	HER2	Cleavable Maleimide tetrapeptide linker	Dxd	AstraZeneca/ Daiichi Sankyo	2019
9	Trodelvy	Sacituzumab govitecan	1. Locally advanced or metastatic triple-negative breast cancer 2. Locally advanced or metastatic urothelial cancer	Trop-2	Cleavable Carbonate linker	SN-38	Immunomedics	2020
10	Blenrep	Belantamab mafodotin	Relapsed or refractory multiple myeloma	BCMA	Noncleavable Maleimidocaproyl linker	MMAF	GlaxoSmithKline	2020
11	Zynlonta	Loncastuximab tesirine-lpyl	Relapsed or refractory large B-cell lymphoma	CD19	Cleavable Valine-alanine linker	Pyrrolobenzodiazepine (PBD) dimer	ADC Therapeutics	2021
12	Tivdak	Tisotumab vedotin-tftv	Recurrent or metastatic cervical cancer	Tissue factor	Cleavable Valine-citrulline linker	MMAE	Seagen	2021

development. Over 85 bispecific antibodies are currently in clinical development, ∼86% of which are being evaluated in patients with cancer (Labrijn et al., 2019).

2. Miniaturized Antibody. ADCs are designed to concentrate in specific tumor tissues, but various pharmacokinetic challenges narrow their tumor-specific distribution. A critical parameter in this regard is tissue penetration. A complex relationship exists between internalization rate and tissue penetration in that conjugates internalized into cells too quickly may penetrate tumor tissues more poorly. A recent study of prostate cancer–specific ADCs found that tissue penetration (rather than cell internalization) directly correlated to therapeutic efficacy. As a result, an ADC with higher penetration but lower in vitro potency showed higher in vivo efficacy (Nessler et al., 2020). This study also found that a smaller size and slower internalization time enabled higher tissue penetration and more cell killing in vivo (Nessler et al., 2020).

Miniaturized antibodies are a class of natural antibody-derived antigen-binding fragments, with varying degrees of structural reduction on the variable light chain, variable heavy chain, or constant domain (Jovčevska and Muyldermans, 2020). These antibody fragments retain the antigen-binding ability of the antibody but have decreased size to increase tumor penetration. Some nanobodies (heavy chain–only antibody fragments), such as caplacizumab, ozoralizumab, and vobarilizumab, have entered clinical research. They have shown unique advantages against brain tumors and central nervous system pathologies because of the nanoscale dimensions of approximately 3 nm (Jovčevska and Muyldermans, 2020).

3. Homogeneous Preparation. The development of homogenous ADCs is important because heterogeneity can impact release rates and therefore toxicity.

Historically, this has been challenging because chemical conjugation methods typically result in a range of payloads linked to antibodies at various attachment sites. One way to facilitate site-specific modifications and thereby homogeneous preparation is to encode unnatural amino acids (unAAs) into antibodies. The unAAs, commonly applied through amber stop codon suppression, contain unique functional moieties that allow specific, precise, and efficient protein conjugation chemistry (Roy et al., 2020). Alternatively, antibody site-directed mutagenesis can also improve homogeneous preparation. For example, incorporating an additional cysteine residue into the heavy chain of humanized monoclonal antibodies can take advantage of cysteine reactivity (Wang et al., 2020a). Protein semisynthesis, such as expressed protein ligation, is another powerful tool to generate site-specific modified ADCs (Frutos, 2020). In this method, a peptide can be ligated to an antibody at a specific position via a disulfide bond. In addition, a recent publication presented an antibody self-assembly method. After generating antibody heavy chains and payload-conjugated light chains, the heavy and light chains could self-assemble into a correctly folded antibody to produce a homogenous ADC (Farràs et al., 2020).

4. Acid Switchable Antibodies. A recently reported "acid-switched" antibody showed high target affinity in plasma and the extracellular microenvironment (near physiologic pH). This antibody had a 250-fold weaker affinity within the tumor cell endosome or lysosome (acidic pH), which increased lysosomal delivery, decreased efflux rate, improved payload release, and displayed higher therapeutic efficacy in a xenograft model (Kang et al., 2019). This design was based on the finding that the affinity of the human epidermal growth factor receptor 2 (HER2)-targeting antibody pertuzumab changed with varying pH.
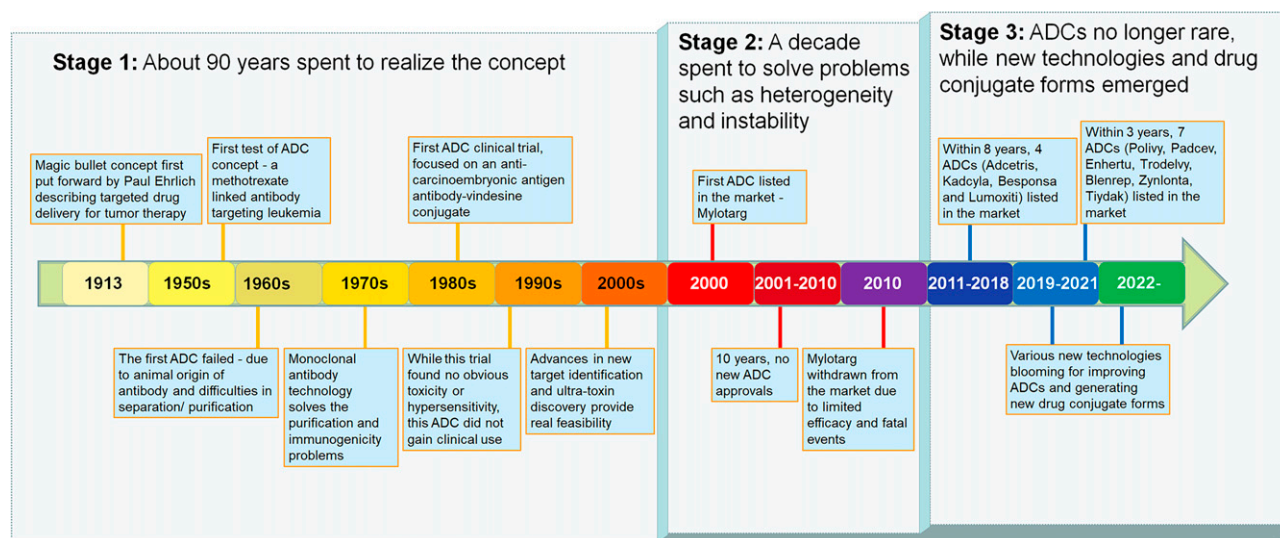
Fig. 1. History, progress, and research stages of drug conjugates.
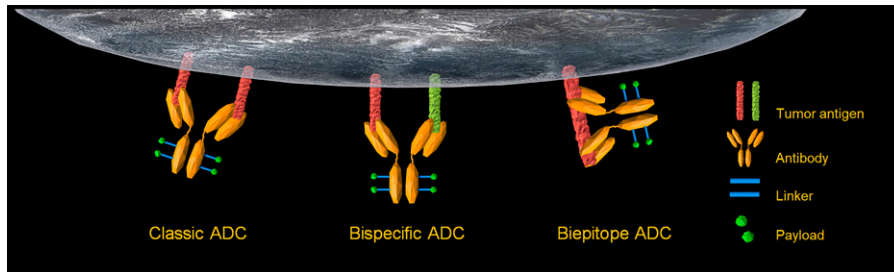
Fig. 2. ADC antibody engineering to optimize internalization rate. Classic ADC antibodies have two of the same antigen recognition sites that can recognize and bind to two molecules of the target epitope at the exact location on each molecule. The internalization rate is mainly determined by the properties of the antigen. Bispecific antibodies have two different antigen recognition sites that can recognize and bind to two different target antigens: one is a tumor target antigen with low internalization, and the other is an antigen with high internalization. The biepitope antibodies have two different antigen recognition sites that can recognize and bind to two different epitopes on the same antigen molecule. Antibody binding to different epitopes on one antigen may change the internalization rate and promote internalization and/or penetration of the ADC into tumor cells.

B. Linker Technology

1. Overall Novel Linker Technologies. The linker attaches the payload to the antibody. Current linkers in clinical ADCs can be classified as cleavable or non-cleavable according to whether they can release their payloads in the tumor cell cytoplasm (Table 1) (Lu et al., 2016). Cleavable linkers are degraded in tumor cells due to changes in environmental factors such as pH, reduction-oxidation (redox) reaction status, and expression of relevant enzymes (Lu et al., 2016).

Decades of effort have been invested into developing these two types of linkers. Current research focuses on using linker technology to achieve homogeneous ADC preparations, developing linker technologies that can easily connect antibodies to their payloads, and identifying new ways to increase DAR while maintaining homogeneity (Fig. 3).

Excluding the intrachain disulfide bond, a typical IgG antibody has four interchain disulfide bonds that, when cleaved, can introduzce eight sulfhydryl groups.
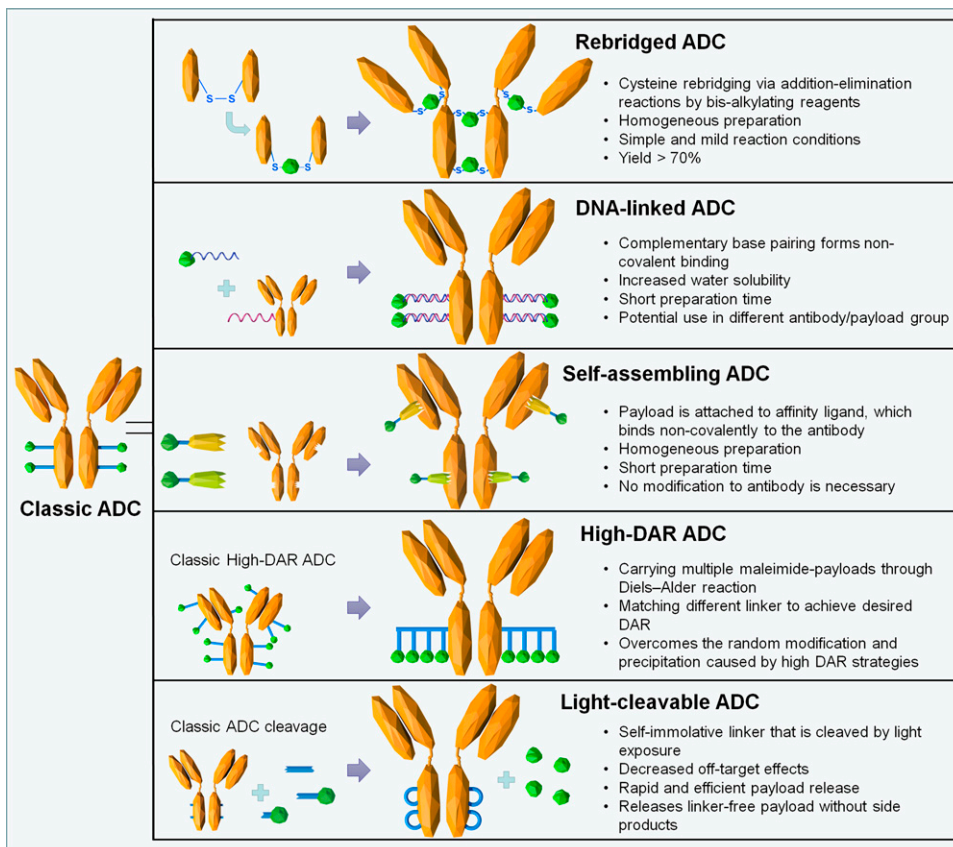
Fig. 3. Schematic diagram and features of some new linker technologies tested in ADC design.

The resulting sulfhydryl groups are then available to attach payloads (Yamada and Ito, 2019). This cysteine rebridging strategy applies bis-alkylating reagents and addition-elimination reactions in mild aqueous conditions and, importantly, is a homogeneous ADC preparation method (Bird et al., 2020). The first rebridging strategy attached monomethyl auristatin E (MMAE) to trastuzumab with a DAR of precisely four (Badescu et al., 2014). Using rebridging, a novel divinylpyrimidine linker platform was reported to have advantages such as a fast conjugation reaction, a covalent bond, a consistent DAR, and a facile linker synthesis compared with normal alkylation-based rebridging (Walsh et al., 2019).

Linkers designed to release payloads due to pH change, redox reaction, or enzymatic activity are all dependent upon endogenous environment changes (Bargh et al., 2019). On the other hand, exogenous stimuli, such as light, can also trigger payload release (Zang et al., 2019). For example, one light-mediated cleavage strategy relies on a photo-caged linker and a cascade addition-elimination reaction on the ε-amine of a lysine residue generated by light exposure at the tumor site (Zang et al., 2019). The advantages include low off-target effects (high physicochemical circulation stability, controlled release position, and no additional compound release) paired with rapid and efficient payload release. However, this approach is limited to areas of the body that can be effectively exposed to light.

Conjugate preparation commonly applies chemical reactions and covalent bonding to attach the payload. However, high-affinity noncovalent binding can also be used in linking technology (Gupta et al., 2019). One antibody has several specific, recognizable regions that can be bound by a ligand or another antibody. A ligand that already contains a payload can attach to the antibody and noncovalently self-assemble on specific, conserved amino acid residues in the antibody to form the ADC. This strategy is also a homogeneous preparation method that can be enacted quickly and increase plasma stability of the ADC (Gupta et al., 2019).

Antibody-oligonucleotide drug conjugates are a new type of ADC derivative, for example trastuzumab-DNA-MMAE (Dovgan et al., 2020). In these conjugates, an oligonucleotide-linked payload and an oligonucleotide-linked antibody can combine to form an antibody-DNA-drug conjugate in a short time and under mild conditions based on hybridization of the oligonucleotides (Dovgan et al., 2020). The presence of the oligonucleotide in antibody-oligonucleotide conjugates can also improve water solubility and decrease precipitation in blood.

Several homogeneous preparation methods have been developed, but most only produce ADCs with a DAR of two (Kumar et al., 2020). Higher DAR approaches may improve therapeutic efficacy. One branched linker strategy can supply a DAR of four, six, or eight. The linkers in this series were connected to antibody cysteine residues by a sulfhydryl-specific iodoacetyl linkage and carried payloads linked by cyclic diene through the Diels-Alder reaction (Kumar et al., 2020). Additionally, another strategy involves the incorporation of unAAs containing dienes into the antibody. This allows for Diels-Alder cycloaddition reactions, providing a rapid and convenient method for homogeneous ADC preparation (St Amant et al., 2018). One example of this strategy incorporates an unAA containing a spiro ([2.4]hepta-4,6-diene structure) or a cyclopropene derivative into the antibody. These antibodies could be coupled with a dihydropyridazine linker-payload via the Diels-Alder reaction in quantitative one-step reactions (Oller-Salvia et al., 2018; St Amant et al., 2019).

MMAE is a common payload in clinical ADCs such as Adcetris, Polivy, and Padcev. Current ADCs utilizing MMAE are constructed using cleavable linkers. Their main shortcoming is an early release of MMAE by cathepsin B, an enzyme ubiquitous in most mammalian cells, which causes safety concerns that offset the advantages of their bystander effect (Wang et al., 2020b). Based on this, one ionized L-cysteine-linker-MMAE noncleavable ADC was prepared and showed comparable cytotoxicity, bystander toxicity, and a maximum tolerated dose similar to the unconjugated antibody, an exciting innovation (Wang et al., 2020b).

2. Linker Optimization. Usually, chemical linkers consist of three parts: (1) the antibody-linker adapter, (2) the payload release structure (for cleavable linkers), and (3) the linker-payload adapter (Su et al., 2021). In recent years, the chemical structure of existing linkers has been optimized, focused on rapid connection, improving plasma stability, improving hydrophilicity, and increasing payload release rate. N-succinimidyl-4-(maleimidomethyl) cyclohexanecarboxylate is the most commonly used structure in ADC linkers as an antibody-linker adapter. Dovgan et al. (2016) proposed a 2-(maleimidomethyl)-1,3-dioxane linker as an alternative (shown in Fig. 4). The 2-(maleimidomethyl)-1,3-dioxane is more hydrophilic, with a calculated LogP value of −1.32 compared with 0.35 for 4-(maleimidomethyl) cyclohexanecarboxylate. Meanwhile, 2-(maleimidomethyl)-1,3-dioxane exhibited higher human plasma stability (3% degradation in 120 hours) than 4-(maleimidomethyl) cyclohexanecarboxylate (38% degradation in 120 hours).

Among marketed ADCs, three applied acid-cleavable linkers (a hydrazone linker in Mylotarg and Besponsa and a carbonate linker in Trodelvy). It can be challenging for acid-cleavable linkers to achieve high cytotoxicity because high DAR often increases clearance (Wang et al., 2019). In addition, the half-lives of clinical acid-cleavable linkers are less than
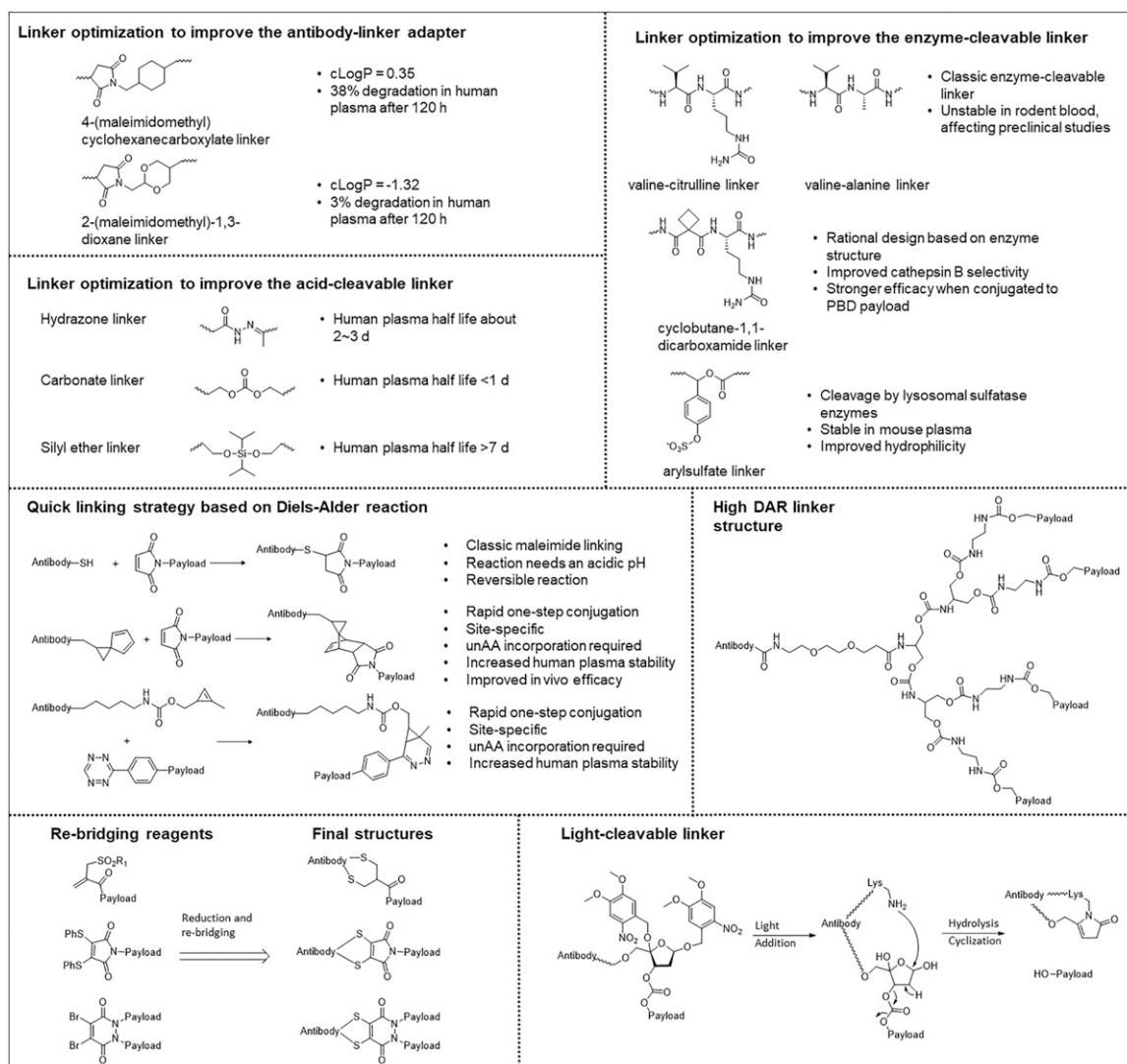
Fig. 4. The chemical structures and characteristics of new linker technologies mentioned in this section.

3 days. In light of this, Wang et al. (2019) designed a silyl-ether acid-cleavable linker. After it was conjugated to an antibody and MMAE to form an ADC, the ADC had a half-life over 7 days and an average DAR of 5.5 and showed quick MMAE release in acidic pH, strong tumor inhibition in vivo, and a good animal hematology safety profile.

Enzyme-cleavable linkers, like valine-citrulline and valine-alanine dipeptides, are the most widely used cleavable linkers. Among approved ADCs, Adcetris, Polivy, Padcev, and Tivdak apply valine-citrulline as their protease-cleavable linker, Zynlonta applies valine-alanine, and Enhertu applies a tetrapeptide. However, peptide linkers often feature two drawbacks. First, they can be unstable in rodent blood due to the Ces1C hydrolase enzyme in their plasma. This limits preclinical studies. Second, their hydrophobicity can hinder the conjugation of payloads and antibodies (Bargh et al., 2020). To address this problem, Bargh et al. (2020) reported an arylsulfate-containing

linking strategy, which can be cleaved by lysosomal sulfatase enzymes, has stability in mouse plasma, and has suitable water solubility.

Lysosomal cathepsin B is an important protease for peptide linker cleavage and payload release, and it is related to cancer progression, so higher cathepsin B specificity means higher efficacy and lower payload release outside of tumor tissue by other cathepsins (Wei et al., 2018). Based on this, one cyclobutane-1,1-dicarboxamide-containing linker was designed to increase cathepsin B selectivity through rational design based on enzyme structure. The cyclobutane-1,1-dicarboxamide-containing linker can be viewed as a valine-citrulline linker analog. When conjugated with MMAE, it showed similar antitumor activity compared with the valine-citrulline-MMAE ADC. Still, when conjugated to PBD, it showed more potent tumor inhibition than the valine-citrulline form (Wei et al., 2018).

3. Site-Specific Conjugation. In site-specific ADC preparation, enzymatic transfer may be a helpful tool

(Anami and Tsuchikama, 2020). This method does not require unAA incorporation or complicated multistep reactions but does require substrate-like recognizable reaction positions on both the antibody and the linker. Microbial transglutaminase and sortase A are two representative enzymes that have been investigated (Dickgiesser et al., 2020; Spidel and Albone, 2019). Chemical-based site-specific conjugation is also in development. Its main advantage relative to enzymatic approaches is faster coupling. One study reported a homogenous conjugation method based on vinyl- and alkynyl-pyridine reagents, which allows a quick reaction, introduces a +1 charge to the antibody, features a precise DAR of 2, and is selective to cysteine residues (Matos et al., 2019). Maleimide reagents are the most common strategy for antibody cysteine conjugation; however, this method has several limitations, including the need for a slightly acidic pH for reaction to avoid lysine residue crossreactivity, the lack of excess equivalents to achieve complete conversion, and, importantly, a reversible reaction. The maleimide conjugation product can undergo the retro-Michael addition reactions to release the maleimide scaffold with biologic thiols in plasma. One study used carbonylacrylic reagents to address this problem. This approach has advantages of cysteine selectivity, mild reaction conditions, rapid kinetics, easy synthesis of payloads, low reagent equivalents, and, importantly, more stable products relative to maleimide conjugations (Bernardim et al., 2019).

Tyrosine residues exposed on antibody surface, mainly in the complementarity-determining region without influence on antigen binding, can also be used for site-selective conjugation. Bemardim et al. (2019) designed a click reaction strategy on tyrosine residues with an N-methylated luminol derivative through a single-electron transfer reaction for drug conjugate preparation without genetic encoding. They applied this conjugation method to trastuzumab, showing it had the advantages of a short reaction time, few byproducts, and a resulting half-life of 20 days (Sato et al., 2020).

C. Payload Technology

Payloads are core components that mediate the antitumor effect. Typically, ADCs release payloads intracellularly after lysosomal processing or following a change in the environment to a characteristic such as redox potential. High potency is of critical importance because the delivery mechanisms of ADCs limit the amount of payload molecules that can enter the tumor cell, in exchange for improved tumor selectivity. Payloads bind to critical targets such as microtubules or genomic DNA to inhibit tumor cell proliferation (Anderl et al., 2013). Research on ADC payloads is ongoing to develop more potent payloads, reveal further mechanisms of current payloads, and identify new payload targets.

1. Mechanism Studies. Mechanism studies of ADC payloads (Fig. 5), such as identifying their binding targets, understanding their intracellular processing, and their tumor resistance mechanisms, offer opportunities for designing higher potency derivatives (Hafeez et al., 2020). Concerning the binding targets, one study detailed the mechanism of an indolinobenzodiazepine dimer, a payload found in several ADCs that are in clinical trials. Through indolinobenzodiazepine-DNA-binding ELISA and evaluation of indolinobenzodiazepine analogs, the investigators revealed that the indolinobenzodiazepine dimer has a similar structure as a pyrrolobenzodiazepine dimer. However, it acts through a different mechanism in that the pyrrolobenzodiazepine dimer crosslinks DNA, whereas the indolinobenzodiazepine dimer alkylates DNA. Both monoimine and diimine indolinobenzodiazepine dimers could bind to DNA to form an adduct that induced nuclease cleavage (Singh et al., 2020b).

The intracellular processing of ADCs can also impact their potency. A CRISPR-Cas9 screening approach was reported that illustrated mechanisms and identified regulators in ADC intracellular trafficking and activation. For example, it found that C18ORF8/RMC1 is a critical ADC toxicity regulator affecting endosomal maturation, a subset of late endolysosomal regulators that influence ADCs with noncleavable linkers. Also, it found that sialic acid depletion enhanced T-DM1 lysosomal delivery and killing in diverse cancer cell types (Tsui et al., 2019).

Other mechanism studies have focused on tumor resistance mechanisms. During the ADC treatment of cancer, tumor cells gradually generate multiple different types of resistance, including downregulation of the surface target, altering intracellular ADC trafficking, reducing lysosomal enzyme activity, and overexpressing drug efflux transporters (Collins et al., 2019). T-DM1 has been extensively studied with respect to the development of such resistance mechanisms (Sung et al., 2018).

2. Payload Optimization. Adjustment of physical and chemical properties, especially polarity, of current payloads can overcome some problems faced during ADC preparation and application. Hydrophobicity of payloads limits the maximum DAR number, affects the bystander effect, and impacts plasma stability (Buecheler et al., 2018). Attaching too many hydrophobic payload molecules changes the conformational stability of an antibody. This increases its tendency for aggregation and precipitation, and eventually affects the maximum DAR. Hydrophobic payloads can easily penetrate cell membranes and kill surrounding antigen-negative tumor cells through the bystander effect. In contrast, hydrophilic payloads are slower to diffuse out of the cancer cells and have reduced bystander impacts. Though pyrrolobenzodiazepine dimers offer high cytotoxicity, they also are hydrophobic and affect antibody stability. Thus, one study introduced a host-guest chemistry approach that improved the physicochemical

properties of pyrrolobenzodiazepine dimer payload by using a capsule named CB[8] to encapsulate 12-mer polyethylene glycol (PEG) harboring a methyl viologen moiety at one terminus (MV-PEG12) together with a pyrrolobenzodiazepine dimer harboring an indole moiety at the C2' position (SG3811) to form an ADC. The hydrophilic PEG balanced the hydrophobic payload and improved overall ADC stability (Sonzini et al., 2020). This formulation approach masked the hydrophobicity of SG3811 and improved the ADC physical stability without loss of potency related to chemical modification (Sonzini et al., 2020).

3. Multiple Payloads on One Antibody. Most systemic cancer chemotherapy regimens apply multiple drugs, but clinical ADCs contain only one single-drug payload. Chemotherapy drugs used in a combination treatment often have different mechanisms of action to provide better outcomes. ADCs that contain multiple payloads are not common, but their application has been proposed to slow development of drug resistance in cancer cells. One study reported a dual-payload, HER2-targeting ADC (linked at engineered site-specific selenocysteine/cysteine residues) containing a DNA crosslinking agent PNU-159682 and a tubulin polymerization inhibitor monomethyl auristatin F (MMAF) (Nilchan et al., 2019). In vitro, this dual-payload ADC showed similar potency against HER2-expressing cell lines as did a single-PNU-159682 ADC but stronger potency

than a single-MMAF ADC. Mechanism-of-action studies confirmed the dual mechanism of this ADC. PNU-159682 causes S-phase cell cycle arrest due to its DNA-damaging activity, whereas MMAF inhibits tubulin polymerization to cause G2/M-phase cell cycle arrest simultaneously, exerting a dual-killing mechanism in cancer cells (Nilchan et al., 2019).

Other dual-payload ADCs include a dual-pyrrolobenzodiazepine (PBD) dimer/MMAE ADC (where MMAE functions as a tubulin polymerization inhibitor and PBD functions as a DNA minor groove alkylator) (Kumar et al., 2018), a dual-MMAE/MMAF CD30-targeting ADC (with both cleavable linker and noncleavable linker to kill neighboring antigen-negative cancer cells) (Levengood et al., 2017), and a dual-α-amanitin/MMAE ADC (where α-amanitin functions as an RNA-polymerase II inhibitor) (Świderska et al., 2018). Two different payloads with two unrelated mechanisms may achieve synergistic action and minimize the chance of cancer resistance. The engineered antibody platform can be used for conjugating any desired payload to form dual-payload ADC. At the same time, achieving synergy often requires different concentrations of each drug, and it is presently unclear how dual-payload ADCs would maximize the synergism ratio.

4. New Payload Development. New payload development is always an attractive field for ADC design and tumor chemotherapy. New scaffold discovery is
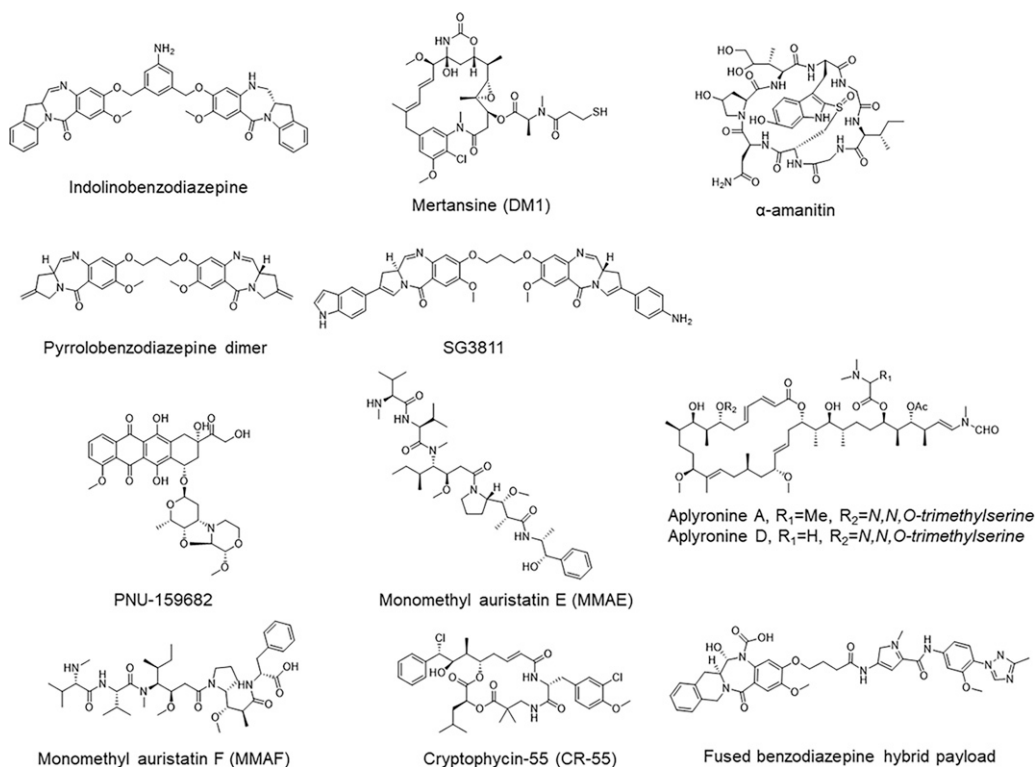

Fig. 5. The chemical structures of payloads mentioned in this section.

challenging and requires a lot of work in areas such as natural product identification, high-throughput screening, or structure-based drug design, which need decades of research (Gromek and Balunas, 2015; Wang et al., 2017). Comparably, structural modification of approved payloads or well-researched toxins is faster to identify new payloads. For example, cryptophycin-52 is a tubulin inhibitor with promising antitumor activity at picomolar levels, but its narrow therapeutic window and lack of a coupling site hindered further development. Recently, an ADC was generated based on a cryptophycin-52 derivative containing a cryptophycin-55 prodrug with a free hydroxyl group. This ADC showed promising results including nanomolar IC_{50} values in $HER2^+$ tumor cell lines, potent activity in mouse xenograft models, and successful intracellular release of epoxidized cryptophycin-52 (Lai et al., 2020). Additionally, cryptophycin and its derivatives have advantages of high potency, hydrophilicity, and lack of P-glycoprotein susceptibility (Verma et al., 2015). Some new compounds that can bind to two targets as dual-targeting warheads have also been reported, such as aplyronines that simultaneously target actin and tubulin to disrupt cytoskeletal dynamics (AnŽiček et al., 2018). Aplyronines are marine-origin antimitotic macrolides with picomolar cytotoxicity. One unprecedented hybrid payload was designed, which had a tetrahydroisoquinoline-fused benzodiazepine ring system combined with surrogates of (1-methyl-1H-pyrrol-3-yl)benzene structure (shown in Fig. 5). Computational modeling on DNA identified its structure-activity relationship showing a similar binding region as the PBD dimer. Good inhibition of lung (H226), gastric (N87), ovarian (OVCAR3), and colon (HCT116) cancer cell lines was observed, as well as a strong tumor regression in an N87 gastric cancer xenograft model with a single dose of 10 nmol/kg in the form of an ADC (Sivaprakasam et al., 2021).

One review highlights total synthesis methods of several payload toxins including calicheamicin, uncialamycin, N^{14}-Desacetoxytubulysin H, trioxacarcins, epothilone, thailanstatin, disorazoles, shishijimicin, and namenamicin (Nicolaou and Rigol, 2019). This research offers potential payload backbones (synthetic intermediate products) and useful synthetic strategies and further enlightens the development of new payloads with higher potencies and lower synthetic complexities.

D. Drug Conjugate Analysis Technologies

With the number of ADCs being designed, comprehensive ADC analysis technologies are also being developed. They aim to supply faster analysis relative to traditional approaches and provide more detailed information on the critical issues of conjugation monitoring, DAR determination, conjugate stability, and metabolism to reduce time-to-clinic.

1. Monitoring of Coupling Reactions. The linking process, often a coupling reaction, is a major step for drug conjugate manufacturing. Common monitoring methods for this step include separation by chromatography and detection with UV/Vis spectroscopy or mass spectrometry (MS). The establishment of detailed kinetic models for the conjugation reactions can find influencing factors, improve conjugation yields, and cut preparation time and cost. For several ADCs, maleimide-based conjugation is essential for the chemical linking of the antibody with a payload (Sun et al., 2017). One study constructed several kinetic modes for this reaction and screened the best model through experimental data set crossvalidation (Andris et al., 2019). In the best model, the R^2 of prediction reached 0.978. This model found that attachment of the first drug molecule would influence the attachment dynamics of subsequent payload molecules. Different salts also influenced the reaction rates (Andris et al., 2019). The modeling results improve understanding of the process of ADC maleimide conjugation. Combining process analytical technologies for reaction monitoring with kinetic models could be a powerful tool. For example, one study used UV/Vis spectroscopy to monitor the ADC conjugation reaction in real time. This method relied on partial least squares regression. It crossvalidated (Q2 > 0.975 in models) and was further validated by an independent prediction set (Andris et al., 2018).

2. Stability Determination. After ADC preparation, it is necessary to measure stability, including antibody conformational changes, aggregation tendency, and half-life, (Buecheler et al., 2020) because these parameters contribute to the potential side effects and inactivation mechanisms. The introduction of the linker and payload can alter the conformation of the antibody locally or globally and sometimes cause aggregation and precipitation in plasma (Ross and Wolfe, 2016; Buecheler et al., 2020). In-depth peptide mapping combined with a protein conformational footprint assay is one method to address this question (Fu et al., 2020). It obtains a snapshot of the ADC structural conformation and stability profile, which is a quick and convenient way to measure the "fitness" of the ADC (Fu et al., 2020). Comparing an antibody profile before and after conjugation identifies the aggregation tendency. Stability or aggregation tests of ADCs can directly reflect aggregation tendency and intrinsic effectiveness and potential immunogenicity. However, at present, real-time identification and quantification test methods remain inaccessible. One label-free method based on the combination of Raman spectroscopy and a support vector machine–based regression model combination was reported as a solution to real-time analysis of ADC stability (Zhang et al., 2019a). This method can precisely differentiate aggregation levels of antibody-like samples pre- and

postisothermal incubation. Importantly, it can be used as an inline analytical tool and for studies on aggregation mechanisms (Zhang et al., 2019a).

3. Drug Antibody Ratio Determination. The DAR is a critical attribute of ADC quality. Hydrophobic interaction chromatography has been a gold standard for the DAR analysis of ADCs, and in recent years, high-resolution mass spectrometry has also proved to be a feasible way to determine DAR (Li et al., 2020). The combination of hydrophobic chromatography with mechanistic modeling can reportedly separate ADCs with different DARs and present scenarios of quantified ADCs with different linker chemotypes and varying DARs and thus determine the DARs (Andris and Hubbuch, 2020). Native size-exclusion chromatography-mass spectrometry can also be used in DAR quantification. One study used this approach to quantify ADCs with three different linker chemotypes and DARs ranging from 2 to 8 (Jones et al., 2020). In addition, size-exclusion chromatography-mass spectrometry data are also a bridge to hydrophobic interaction chromatography data without a correction factor or offset.

4. Metabolite Analysis. Metabolism, including varied DAR, aggregation, and degradation, during and after administration needs to be monitored to ensure the safety and efficacy of ADCs. A recent liquid chromatography-MS/MS method was designed for rapid online analysis of reduced ADCs, suitable for analyzing ADC fragments, such as a light chain without payload, a light chain with one payload, a heavy chain without payload, and a heavy chain with one to three payloads. This could be performed within one hour and did not require a conventional enzymatic digestion step, which is time consuming and may introduce artifactual modifications (Larson et al., 2020). An ultra–high-performance liquid chromatography-MS method was reported to measure free and total SN-38 and its glucuronidation metabolite (SN-38G) of sacituzumab govitecan through isotope dilution. This approach has good linearity ($r^2 \geq 0.997$), accuracy (relative error $\leq \pm 9.1\%$), precision (CVs $\leq 7.7\%$), and extraction recoveries (85.6–109.3%) as its advantages (Pandey et al., 2020).

III. New Targets of Drug Conjugates

Drug conjugate development benefits from the discovery of new tumor-specific targets (tumor cell surface antigens). The marketed ADCs target several well-studied tumor antigens, such as CD33, CD30, and HER2. New tumor antigens have been discovered (Razzaghdoust et al., 2021) (Table 2). Their original genes, distribution in human tissues, distribution in the human population, and endocytosis activity have been studied in varying depths, providing new foundations for ADC development and targeted therapy agents (Boni et al., 2020).

A. New Drug Conjugate Targets

1. New Targets Examined in Preclinical Studies. The development of agents targeting new tumor antigens is essential in part because current agents do not have broad cancer-type coverage. For example, the HER2-targeted ADCs including Kadcyla and Enhertu are in clinical use; however, HER2$^+$ breast cancers only represent about 25% of total breast cancers (Gandullo-Sánchez et al., 2020). To address this limitation of target expression, alternative targets such as human epidermal growth factor receptor 3 (HER3) may be able to fill the vacancy, but no HER3-targeted therapy is currently clinically available. Several HER3-targeted ADCs are in development and preclinical studies, such as U3-1402 and EV20 as shown in Table 2. These have shown promising results like complete and long-lasting tumor regression, over 300 days, in murine models (Hashimoto et al., 2019; Gandullo-Sánchez et al., 2020).

B7-H3 (CD276) is a B7 family protein with immunoregulatory functions, which is overexpressed on many solid cancers and associated with disease severity/survival. One study examined an ADC with a cleavable Val-Cit linker and duocarmycin hydroxybenzamide azaindole payload against B7-H3 in preclinical tumor models of breast, ovarian, and lung cancer. It induced a 98% tumor volume reduction and 4 of 5 complete regressions following a single-dose administration and achieved the expected pharmacokinetic and safety profile in cynomolgus monkeys (Scribner et al., 2020).

Triple-negative breast cancer (TNBC) lacks three important receptors (i.e., estrogen receptor, progesterone receptor, and HER2) compared with other types of breast cancer. TNBC is more aggressive and likely to relapse, and, importantly, it needs an efficient target for treatment. He et al. (2020) generated an aptamer-drug conjugate, AS1411-triptolide conjugate, targeting nucleolin through AS1411, which showed high cytotoxicity to the MDA-MB-231 cell line, increased efficacy in the TNBC mice model in vivo (67% tumor size reduction), and no apparent side effects to healthy organs, Similarly, antigens including anaplastic lymphoma kinase, CD205, and trophoblast glycoprotein (5T4) were also tried as ADC targets in tumor cell lines and animal tumor models (summarized in Table 2).

2. Widely Expressed Antigens as Targets. Some tumor antigens are expressed on the cell surface of many tumor types (von Bergwelt-Baildon et al., 2011). Such widely expressed antigens include c-KIT, nectin-4, c-Met, insulin-like growth factor type 1 receptor (IGF-1R), and death receptor 5. ADCs targeting these antigens can act as broad-spectrum antitumor agents to treat multiple tumor types (Andersen and Thor, 2002). Such ADCs have been tested in

TABLE 2
Overview of selected preclinical studies of new targets and associated ADC development

Target	Cancer	ADC Name	Payload	In Vitro Effect	Tumor model	In Vivo Effect	Reference
HER3	Overexpressed on various cancers (breast, gastric, and ovarian cancers and melanoma)	U3-1402	DXd	Efficient internalization and payload release in HER3$^+$ cell lines (HCC1569, SK-BR-3, MDA-MB-175VII, MDA-MB-453, MDA-MB-361, and OVCAR-8)	Human breast cancer cell line and patient-derived breast cancer xenograft mice	1. Antitumor activity dependent upon HER3 expression level 2. Tolerable safety profiles in rats and monkeys	Hashimoto et al., 2019
HER3	Overexpressed on various cancers (breast, gastric, and ovarian cancers and melanoma)	EV20	MMAF	Efficient proliferation inhibition in cell lines resistant to anti-HER2 therapies (BT474 cell)	Trastuzumab-resistant HER2$^+$ breast cancer xenograft mice	HER3-dependent complete and long-lasting tumor regression (over 300 days)	Gandullo-Sánchez et al., 2020
Anaplastic lymphoma kinase	The most common somatically mutated gene in neuroblastoma	CDX-0125-TEI	NMS-P945	1. Efficient antigen binding and internalization 2. Cytotoxicity at pM concentrations	Neuroblastoma wild-type and mutant xenograft mice	Dose-dependent antitumor effect and significant tumor growth delay	Sano et al., 2019
CD205	Lymphoma, leukemia, and multiple myeloma	MEN1309/OBT076	DM4	Antiproliferative activity against 42 types of B-cell lymphoma cell lines with a median IC$_{50}$ of 200 pM	TNBC, pancreatic, and bladder cancer cell lines xenograft mice	Complete tumor regression at a dose of 5 mg/kg in all 8 model mice	Eugenio et al., 2020
c-KIT (CD117)	Gastrointestinal stromal tumors, small cell lung cancer, melanoma, non–small cell lung cancer, and acute myelogenous leukemia	LOP628	DM1	Antiproliferative activity on c-KIT$^+$ cell lines (GIST882, GIST430, GIST-T1, Kasumi-1, Kasumi-6, NCI-H526, and NCI-H1048)	Gastrointestinal stromal tumors and small cell lung cancer xenograft mice	1. Superior antitumor activity against imatinib-resistant tumors and complete tumor regression for 130 days when coadministered with imatinib 2. Well tolerated in monkeys (dose of 30 mg/kg every 3 weeks)	Abrams et al., 2018
Nectin-4	Re-expressed on various cancers	N41mab-vcMMAE	MMAE	Dose-dependent cytotoxicity	TNBC primary tumor, metastatic lesion, and local relapse	Rapid, complete, and durable immune responses	M-Rabet et al., 2017
cMet	Amplified, mutated, or overexpressed cMet commonly seen in many human tumor types	TR1801-PBD-ADC	PBD	1. Antitumor activity at picomolar concentration 2. High toxicity to both cMet high-expression and medium-to-low expression cell lines	Patient-derived xenograft mice models	1. Complete tumor regression in 90% of gastric, colorectal, and head and neck cancers 2. Good tolerability in rats (0.5, 1, 1.5, and 2 mg/kg)	Gymnopoulos et al., 2020
cMet	Amplified, mutated, or overexpressed cMet commonly seen in many human tumor types	cIRCR201-dPBD	PBD	Antitumor activity on 47 different cancer cell lines in a cMet expression level dependent manner	cMet-amplified cancer cells xenograft mice models	Significant antitumor activity and complete tumor regression at a high dose of 0.8 mg/kg	Min et al., 2020

TABLE 2—*Continued*

Target	Cancer	ADC Name	Payload	In Vitro Effect	Tumor model	In Vivo Effect	Reference
IGF-1R	Overexpression occurs in numerous tumor tissues	hz208F2-4-W0101	Auristatin derivative	IGF-1R expression-dependent cell cytotoxicity in various cancer cell lines	Mouse models expressing different levels of IGF-1R	Potent tumor regression correlated with IGF-1R expression level	Akla et al., 2020
Death receptor 5	Overexpressed on various cancers	Zapadcine-1	MMAD	1. Rapidly endocytosed into the lysosome of cancer cells 2. Antitumor effect against lymphocyte leukemia cells and solid tumor cells	Patient-derived xenografts of human acute leukemia and cell-derived xenografts of Jurkat E6-1, BALL-1 and Reh	1. Drastically eliminates the xenografts 2. Acceptable safety profile in rat and cynomolgus monkey	Zhang et al., 2019b
AXL	Overexpressed on various cancers and plays important roles in formation, growth, and metastasis of tumors	AXL-107-MMAE	MMAE	Efficient AXL-specific cytotoxicity in various cancer cells	Patient-derived xenografts, including melanoma, lung, pancreas, and cervical cancer	1. Potent antitumor activity 2. Acceptable safety profile in cynomolgus monkey	Boshuizen et al., 2018
DLL3	Overexpressed in neuroendocrine prostate cancer and related to castration-resistant neuroendocrine prostate cancer	SC16LD6.5	PBD	NA	DLL3-expressing prostate cancer xenografts	Complete tumor regression and durable antitumor responses to DLL3 high expression xenografts	Puca et al., 2019
GPC2	Differential expression in neuroblastoma and required for neuroblastoma proliferation	D3-GPC2-PBD	PBD	Cytotoxic to human GPC2 expressing neuroblastoma cell lines	GPC2-expressing neuroblastoma cell line xenografts	1. Efficacy against tumor growth with only a single dose 2. Significantly prolonged survival (80% of mice over 60 days)	Bosse et al., 2017
Cadherin-6	Differential expression in ovarian and kidney cancers	HKT288-DM4	DM4	NA	Human ovarian and renal cancer xenografts	1. Durable tumor regression in ovarian and renal cancers 2. Acceptable tolerability profile in rats and nonhuman primates	Bialucha et al., 2017
GPNMB	Expressed on MAPK inhibitor-treated melanoma cells	CDX-011	MMAE	NA	A375 and WM2664 melanoma cell xenograft model mice	Melanoma regression and delayed recurrent melanoma growth when combined with MEK inhibitors trametinib	Rose et al., 2016
β-1,3-N-acetylglucosaminyl transferase	Overexpressed in breast cancers	gPD-L1-ADC	MMAE	1. Selectively suppressed tumors with PD-L1 antigen 2. Potent cytotoxic and bystander-killing effect to TNBC	PD-L1 expressing mouse and human cancer cell xenograft model mice	1. Complete regression of 4T1-hPD-L1and EMT6-hPD-L1 tumors 2. Significantly better survival (about 75%) than glycosylated PD-L1 treated mice (about 30%)	Li et al., 2018

TABLE 2—Continued

Target	Cancer	ADC Name	Payload	In Vitro Effect	Tumor model	In Vivo Effect	Reference
Protein tyrosine kinase 7	Enriched on tumor-initiating cells in TNBC, ovarian cancer, and NSCLC	PF-06647020	Aur0101	Strong cytotoxicity against H661, H446, and OVCAR3 cancer cell lines	Human tumor xenograft (NSCLC, TNBC, and ovarian cancer) model mice	1. Sustained tumor regression for 200 days in BR22 xenograft and 150 days in most BR31 xenografts 2. Reduced tumor-initiating cell frequency 5.5-fold with respect to control ADC and 2.1-fold with respect to docetaxel	Damelin et al., 2017
EphA2	Comprise a large family of receptor tyrosine kinases, low expression in normal adult tissue, but overexpression in a wide range of solid tumors	BT5528	MMAE	Successfully bind to the surface of HT-1080 cells with high EphA2 expression	Murine xenograft models	Decreased tumor burden (return to baseline values after three dosing cycles) and prolonged survival	Bennett et al., 2020

5T4, trophoblast glycoprotein; Aur0101, an auristatin microtubule inhibitor; AXL, a tyrosine-protein kinase receptor; cMet, tyrosine-protein kinase Met; DM1/DM4, a maytansinoid microtubule disruptor; Dxd, a topoisomerase I inhibitor payload exatecan derivative; EphA2, erythropoietin-producing hepatocellular (Eph) receptor; MMAD, monomethyl auristatin D; NMS-P945, a DNA minor groove alkylating agent.

tumor cell lines and animal tumor models listed in Table 2. For example, the tyrosine kinase AXL has been found in several drug-resistant tumor clones and a broad spectrum of cancer types. One recent AXL-targeted ADC, AXL-107-MMAE, displayed potent antitumor activity and tumor regression in 18 of 25 patient-derived xenografts such as melanoma, lung, pancreas, and cervical cancer (Boshuizen et al., 2018). In the safety study, the tolerated dose of AXL-107-MMAE reached 6 mg/kg in cynomolgus monkeys, where no histopathological changes in the liver, spleen, and lung were observed (Boshuizen et al., 2018). Delta-like protein 3 (DLL3) is expressed in most castration-resistant neuroendocrine prostate cancers (76.6%) (Puca et al., 2019). A DLL3-targeted ADC [rovalpituzumab tesirine (SC16LD6.5)] was designed to treat this type of prostate cancer and found complete and durable responses 35 days after treatment against DLL3-expressing prostate cancer xenografts (Puca et al., 2019), though lack of efficacy in additional clinical trials ultimately led to the failure of this candidate. Furthermore, widely expressed antigens are potential targets for the therapy of resistant tumors.

3. New Target Discovery Methods. New tumor antigens are being identified, and new mechanisms of tumor growth or drug resistance are being discovered, which provide targets for developing new ADCs. For example, through RNA sequencing, glypican proteoglycan 2 (GPC2) was identified as an oncoprotein. Sequencing studies found that this protein had differential expression in neuroblastoma but not in normal childhood tissues and was required for neuroblastoma proliferation. Based on this, a GPC2-targeting ADC, D3-GPC2-PBD, was designed. This ADC showed potent cytotoxicity toward neuroblastoma cells and prolonged overall survival in a mouse model with a single 1 mg/kg dose (Bosse et al., 2017). Bialucha et al. (2017) generated a differential gene expression analysis on genes obtained from normal and cancer samples. A cell-cell adhesion molecule, cadherin-6, was identified as the top candidate in the genome analysis. Cadherin-6 had previously been reported to have significant differential expression in ovarian and kidney cancers. Therefore, they prepared a cadherin-6–targeted ADC (HKT288-DM4), which yielded durable tumor regressions of ovarian and renal cancer xenograft in vivo and also showed a good preclinical safety profile (Bialucha et al., 2017).

Mechanistic studies of tumor gene regulation are also a way to find new targets. After BRAF and MEK inhibitor treatment, the melanosomal differentiation gene glycoprotein non-metastatic melanoma protein B (GPNMB) is induced, and the microphthalmia-associated transcription factor overexpresses, which is associated with poor prognosis. Rose et al. (2016)

demonstrated that microphthalmia-associated transcription factor is required for treatment-induced GPNMB upregulation. Treatment of melanoma with a BRAF and/or MEK inhibitor combined with the GPNMB-targeted ADC CDX-011 caused melanoma regression in preclinical animal models and delayed recurrent melanoma growth more than MEK or BRAF/MEK inhibitor treatment alone.

4. Targeting Tumor Antigens with Biologic Functions. Some tumor antigens have biologic functions in cancer cells including tumor resistance, immune suppression, or tumor metastasis (Schumacher et al., 2019). The development of ADCs against these targets may inhibit tumor growth through multiple mechanisms and achieve an additive or synergistic effect. For example, the enzyme β-1,3-N-acetylglucosaminyl transferase participates in the endothelial growth factor–induced interaction of programmed death ligand 1 (PD-L1) and its receptor programmed death protein 1 (PD-1) in TNBC. Downregulation of β-1,3-N-acetylglucosaminyl transferase could enhance cytotoxic T-cell–mediated antitumor immunity. In addition, PD-L1 antibodies can block the PD-L1/PD-1 interaction and thereby promote PD-L1 internalization and degradation (Li et al., 2018). Based on these, an ADC named scPDL1-DM1 targeting β-1,3-N-acetylglucosaminyl transferase was designed and reported to release the cytotoxic agent and enhance immune checkpoint therapy against TNBC (Li et al., 2018). Tumor-initiating cells are associated with tumor recurrence and metastasis and indicate a poor prognosis, especially in TNBC, ovarian cancer, and non–small cell lung cancer (NSCLC). Protein tyrosine kinase 7 is a highly conserved but catalytically inactive receptor tyrosine kinase in the Wnt signaling pathway. It is enriched in tumor-initiating cells in TNBC, ovarian cancer, and NSCLC (Damelin et al., 2017). An ADC targeting the protein tyrosine kinase 7 was prepared and found to induce sustained tumor regression for 150 days in most tumor xenografts, reduce the frequency of tumor-initiating cells 5.5-fold relative to a control ADC and 2.1-fold relative to docetaxel, and also showed other inhibitory mechanisms including angiogenesis and immune cell stimulation (Damelin et al., 2017).

B. Stromal Targeting

Current conjugates target internalizing receptors on cancer cells, leading to intracellular payload release. However, only a subset of patients with solid tumors has sufficient expression of such a receptor (Lee et al., 2019). It is possible that noninternalized ADCs may better tackle this problem. Moreover, nonmalignant stromal cells, within the tumor microenvironment, transport nutrients to support tumor growth and progression while blocking drug access, causing treatment resistance (Denton et al., 2018; Li and Simon, 2020). These cells can occupy up to 90% of a solid tumor mass (Dvorak, 1986). Stromal cells in

different types of tumors share some identical markers, so it follows that an ADC targeting stromal cells could potentially be helpful for multiple tumor types (Borriello et al., 2017). Therefore, stromal cell–targeted ADCs are worthy of development.

One approach targets specific proteins overexpressed by stromal cells of the tumor microenvironment (Raavé et al., 2018), such as fibroblast activation protein, which is thought to regulate tumor proliferation (Liu et al., 2012). In a study by Ostermann et al. (2008), an antibody targeting fibroblast activation protein was conjugated to maytansinoid as an ADC named FAP5-DM1. It showed long-lasting tumor growth inhibition ($\sim$45 days) in several xenograft cancer models including head and neck carcinoma, lung cancer, and pancreas carcinoma compared with free antibody and vehicle.

Other targets have been identified in the extracellular matrix of the tumor microenvironment. Fibronectin glycoprotein is one of such targets. It is present in ample amounts in many cancer types but is not detectable in healthy cells (Raavé et al., 2018). In one study, the fibronectin extra domain A was targeted with an antibody conjugated to either a maytansinoid derivative (DM-1) or two duocarmycin derivatives. The DM-1–containing ADC (7 mg/kg for 7 days) was able to induce a complete remission (tumor-free for more than 180 days) in immunocompetent mice grafted subcutaneously with F9 teratocarcinoma that had reached a volume of 100 mm^3, without antibody internalization (Perrino et al., 2014).

Collagen type IV also has been explored as a target. Yasunaga et al. (2011b) studied an antibody against this collagen conjugated to SN-38, a topoisomerase I inhibitor with an acid-labile ester linker. In mice bearing SUIT2, stroma-rich pancreatic cancer cells, this conjugate showed an effective tumor growth inhibition at a dose of 3 mg/kg, which was observed after 1 month up to 3 months. Its high molecular mass allowed for extravasation from only the leaky tumor vasculature. Moreover, while bound to its target in the stroma, it showed sustained release and caused damage to both tumor cells and tumor vasculature without significant adverse effects.

In a similar study, Yasunaga et al. (2011a) developed an antifibrin chimeric antibody conjugated to SN-38 via an alkaline-labile ester bond. It was designed to release the drug in the mildly alkaline conditions of the extracellular matrix. The antibody was devised to bind to fibrin and not fibrinogen to prevent the immunoconjugate from forming a complex in the blood. The conjugate selectively accumulated in a stroma-rich skin carcinogenesis model with a dose of 13.3 mg/kg per day, four times weekly. It bound to fibrin clots, where the SN-38 was released

sustainably, inhibited tumor growth for more than 1 month, and damaged tumor vasculature.

Tumor endothelial marker 8 (TEM8) is a highly conserved transmembrane receptor broadly overexpressed on cancer-associated fibroblasts, endothelium, and pericytes. An ADC targeting TEM8 localized to tumor stroma and released MMAE in stromal cells to kill nearby proliferating tumor cells in a target-independent manner (Szot et al., 2018). The anti-TEM8 ADC elicited potent anticancer activity against different types of tumors in mice including human lung, breast, pancreatic, colon, and ovarian tumors and induced bystander effects (Szot et al., 2018). For instance, in mice bearing a pancreatic (HPAC) tumor with TEM8 expression, the ADC shrunk the tumor for ∼80 days. Eleven out of the 14 mice remained tumor free after 180 days with 10 mg/kg 6 injections biweekly. In TEM8$^-$ tumors, regression was observed for ∼30 days, and 9 out of 15 remained tumor free after 180 days, demonstrating the bystander effect. Thus, the single TEM8-targeted ADC showed promise for treating various types of solid cancers. McCann et al. (2018) also reported a TEM8-targeted ADC. In vivo, this ADC bound to tumor stromal cells, internalized, and released MMAE intracellularly. The MMAE was then transported to the extracellular tumor microenvironment via P-glycoprotein, where it could enter solid tumors. This killing mechanism destroys tumor cells while nonmalignant stromal cells remain relatively viable.

A recently reported ADC targeting tenascin-C (an abundant marker expressed on stroma of multiple tumor types) exhibited another strategy for stromal targeting (Dal Corso et al., 2017). This ADC contains a peptide linker that can be broken down by proteases released from dying tumor cells to cleave the payload and kill nearby tumor cells. This ADC, with two doses of 1 mg/kg 3 days apart, inhibited growth of epidermoid carcinoma xenografts for more than 30 days and released the payload into the tumor stroma instead of the cytoplasm (Dal Corso et al., 2017).

Since immune cells are also a part of the tumor microenvironment, one strategy is to use ADCs for targeting them to improve tumor delivery. For instance, an anti-CD25 antibody conjugated to a pyrrolobenzodiazepine toxin was developed (Boni et al., 2020). CD25 is overexpressed in regulatory T cells. These cells can infiltrate the tumor microenvironment, and their absence has been linked to a delayed cancer progression (Arce et al., 2017). CD25 is also overexpressed in Hodgkin and non-Hodgkin lymphomas (Hashimoto et al., 2019). A single dose of 0.6 mg/kg of anti–CD25-pyrrolobenzodiazepine showed a significant improvement in severe combined immunodeficient mice with Karpas 299 subcutaneous tumors, leading to tumor-free survivors over 60 days in 10 of 10 tumor-bearing mice (volume when treated, 100-150 mm^3) compared with brentuximab vedotin as the control (Flynn et al., 2016).

Another approach tested is the simultaneous activation of the immune system and introduction of a chemotherapeutic. In this regard, an anti–PD-L1 antibody was attached to doxorubicin with an acid-labile hydrazone linker. The ADC would not be internalized once bound to the PD-L1 on cancer cells. Thus, doxorubicin is released from the ADC in the acidic extracellular environment, disrupting the tumor microenvironment and facilitating the penetration of PD-L1 into the tumor core (Sau et al., 2019). Rossin et al. (2018) also designed a noninternalizing diabody drug conjugate to target the ovarian and colon tumor microenvironment. This diabody conjugate was administered, then two days later (after ADC accumulation in tumor tissues) a small molecule chemical activator was injected to break the linker via a click reaction and release the payload. This strategy exerted more effective antitumor activity than brentuximab vedotin in vivo (∼30 days of growth inhibition with a dose of 3 and 5 mg/kg). It could hold promise for extending applicable patient populations.

C. Drug Conjugate Repurposing Based on Genetic Testing and Target Expression

Each of the marketed drugs has its own approved indications, but ADCs can be further evaluated against additional indications that express the same target antigen once in clinical use. This strategy can be compelling when combined with genetic tumor profiling that quantifies the expression of the target antigen. For example, trastuzumab deruxtecan was developed to treat HER2$^+$ breast cancer, but in a recent study, it was used to treat HER2$^+$ gastric cancer and exhibited prolonged survival time in these patients (Shitara et al., 2020). Similarly, sacituzumab govitecan is used to treat TNBCs and urothelial cancers because both tumor types can express Trop-2, which is the target of sacituzumab (Tagawa et al., 2021). Enfortumab vedotin was designed for treatment of urothelial cancer by targeting nectin-4. Nectin-4 expression is also found in some types of bladder cancer, which has led to evaluation of this ADC for the treatment of nectin-4$^+$ bladder cancers (Challita-Eid et al., 2016). These examples can be thought of as extensions of ADC indications based on identifying the target antigen expression in distinct types of tumor cells. Considering the individual differences among patients and tumor heterogeneity, it is best to detect the antigen expression level and uniformity of tumor tissues in patients before selecting an ADC treatment scheme. Future treatment paradigms may be less limited by tumor type categorization and more likely selected by individualized tumor genetic profiling.

IV. New Types of Drug Conjugates

A. X-Drug Conjugates (New Drug Conjugate Forms)

Numerous preclinical studies have attempted either to improve therapeutic efficacy or to overcome defects of

current ADCs. These innovations are enlightening the drug conjugate field and broadening the scope of development to include alternative drug conjugate forms. These new forms of drug conjugates include, but are not limited to, small molecule-drug conjugates (SMDCs), antibody fragment drug conjugates, peptide-drug conjugates (PDCs), amphiphilic peptide-drug conjugates, amphiphilic inhibitor-drug conjugates, antibody-polymer-drug conjugates, antibody-photosensitizer conjugates (APCs), ligand drug conjugate (LDCs), and several others (shown in Fig. 6). These are all new drug conjugate designs based on the concept of a "magic bullet" and the clinical success of ADCs.
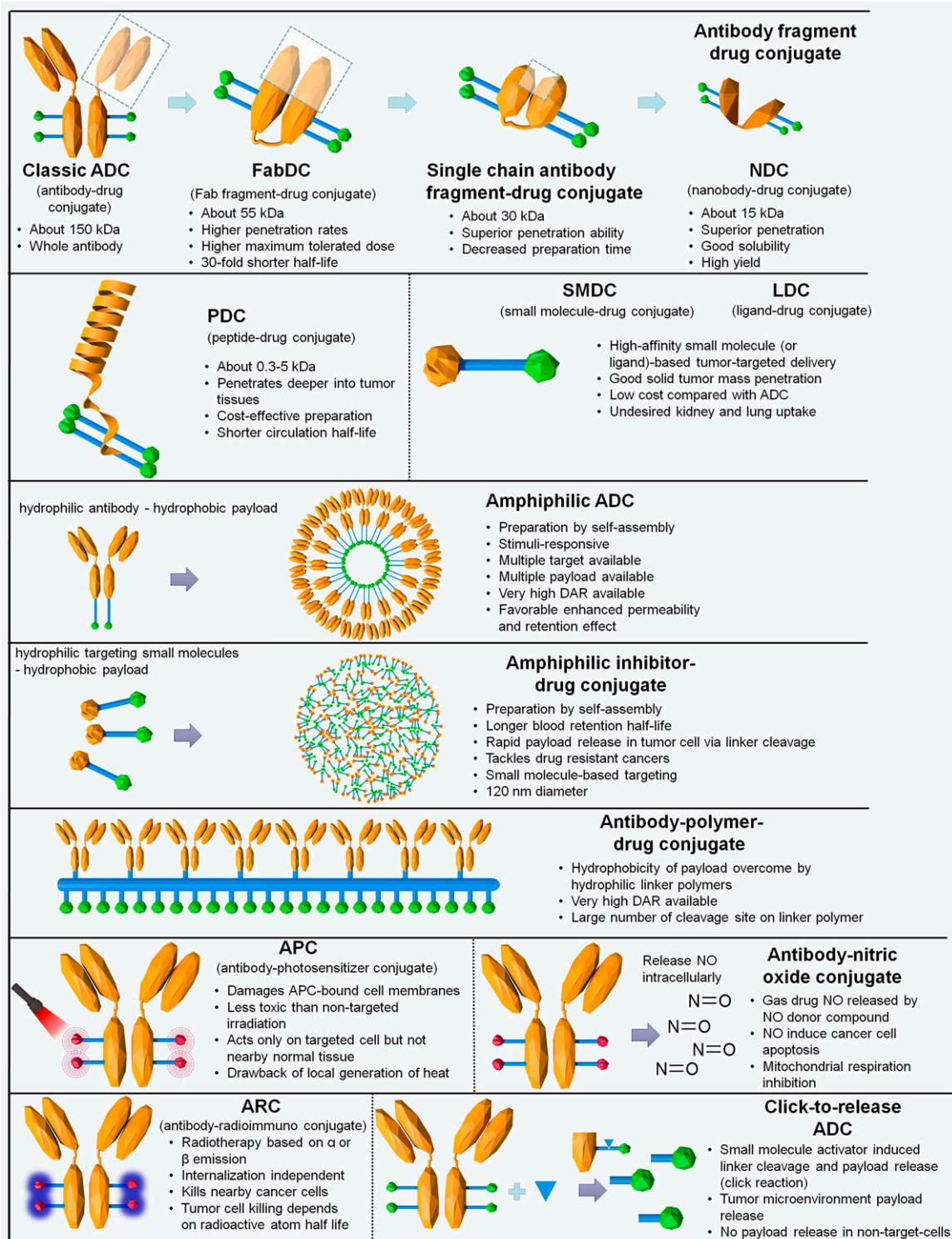

Fig. 6. Schematic diagram and features of some novel drug conjugates tested in preclinical studies.

One problem encountered regarding the therapeutic efficacy of ADCs targeting solid tumors is limited penetrability. Solid tumors are hidden in deep sites and covered with non-neoplastic stromal cells. This can deter the entry of conjugates containing a full-size antibody because of the large molecular mass. Decreasing conjugate size while retaining target-binding activity could improve tumor penetration (Patel et al., 2021). One study compared ofatumumab in an ADC form with it in an antibody fragment drug conjugate form, both of which target lymphoma and contain the MMAE payload. The study found that the Fab fragment-drug conjugate showed an identical IC_{50} value, a higher maximum tolerated dose, a 30-fold shorter half-life, and slightly lower antitumor activity, but most notably a higher penetration rate relative to the full ADC (Liu et al., 2019). Thus, removing the Fc region can boost penetration at the expense of a decreased half-life, but this may be a worthwhile exchange that could be balanced by increased dose frequency.

Phage display technology is an essential tool for screening antibody fragments, such as an scFv, on the external surface of a bacteriophage. It can identify desirable specificity and binding affinities. A recent study constructed an scFv antibody fragment-drug conjugate targeting PD-1 and conjugated with DM1 through a succinimidyl trans-4-maleimidylmethyl-cyclohexane-1-carboxylate linker, yielding potent anti-tumor activity and efficient intracellular trafficking results (Kalim et al., 2020). Single domain antibody fragments (single variable heavy chain or nanobodies) have simpler structures, improved solubility, high yield (50 mg/L) in *Escherichia coli*, and enhanced tissue penetration over full-sized antibodies. At the same time, nanobodies are only ~15 kDa in mass and 2.5 nm in diameter, whereas Fabs are ~55 kDa, both in stark comparison with whole antibodies at ~150 kDa (Sun et al., 2021). One nanobody-drug conjugate was developed for B-cell lymphoma therapy. It showed rapid primary and metastatic lymphoma localization when conjugated with a fluorophore. When conjugated with a payload, the nanobody-drug conjugate controlled tumor growth and metastasis efficiently without apparent systemic toxicity (Fang et al., 2016).

Alternatively, nonimmunoglobulin scaffolds are raising interest for tumor targeting and conjugate design efforts (Merten et al., 2015). Designed ankyrin repeat proteins, adnectins/monobodies, affibodies, and Kunitz domains are some scaffolds with optimal engineering sites and pharmacokinetic properties (Brandl et al., 2020). These scaffolds can enable precisely defined and pharmacokinetically tunable conjugates with any desired polypeptide lengths and half-life; therefore, they allow for the quantitative study of the relationship between linker lengths and half-lives and ADC efficacy in animal models (Brandl et al., 2020).

PDCs are potential candidates based on tumor-targeting peptides including somatostatin, bombesin, cyclic-Arg-Gly-Asp peptide, and aptides (Zhao et al., 2018). They have shorter circulation half-lives compared with full ADCs in vivo. However, a putative coadministration strategy of labeled PDC plus antilabeled antibody was designed using hapten as the label and anticotinine as the antibody. A hybrid complex PDC-antibody formed in vivo, which significantly extended the circulation half-life, tumor penetration, and, ultimately, tumor growth inhibition (Kim et al., 2019). A comparative evaluation of an ADC and a SMDC was conducted. Both forms bind to the same tumor marker, carbonic anhydrase IX, and have the same Val-Cit linker and MMAE payload. The results of in vivo studies showed similar antitumor activity between the ADC and SMDC at the same molar dose. Still, the SMDC had a tumor/blood distribution ratio of ~100:1 six hours after injection, whereas that of the ADC was near 4:7 48 hours after injection (Cazzamalli et al., 2018).

Polymeric nanoparticles are suitable drug carriers, offering advantages including improved drug solubility, prolonged circulation half-life, reduced immunogenicity, controlled release, and enhanced safety (Ekladious et al., 2019). Due to the leaky tumor vasculature, polymeric nanoparticles target the tumor microenvironment by enhanced permeability and retention effect. The combination of nanotechnology with ADC is a new research area. If an amphiphilic peptide-drug conjugate has a hydrophilic target peptide, like Arg-Gly-Asp, and a hydrophobic payload, like DM1, it can self-assemble into nanoparticles due to the amphiphilicity in water via a nanoprecipitation process (Liang et al., 2017). An amphiphilic inhibitor-drug conjugate, containing a hydrophilic anticancer payload like irinotecan, and a hydrophobic inhibitor, like the P-glycoprotein inhibitor quinine, can also self-assemble into nanoparticles and can be used for severe multidrug resistance cancer treatment. In vivo, this amphiphilic inhibitor-drug conjugate was reported to inhibit P-glycoprotein, which pumps irinotecan out of cells, and exhibited high antitumor efficacy (Huang et al., 2019). In addition, antibody-polymer-drug conjugates use a hydrophilic linker polymer connecting to the constant fragment of an antibody and attaching a large number of hydrophobic payloads. The linker polymer contains a variety of cleavage sites, allowing rapid release of all the payload (Wan et al., 2020). An antibody-gold nanoparticle-drug conjugate was reported to deliver high amounts of payloads to targeted cell populations through the gold nanoparticle, which sequestered payloads in hydrophobic pockets. This antibody-gold nanoparticle-drug conjugate successfully penetrated

target tissues as visualized by a transmission electron microscope (Yang et al., 2018).

Antibody-based near-infrared photoimmunotherapy is an attractive strategy for cancer treatment because tumor cells can be selectively and efficiently killed by the targeted delivery of an APC followed by exposure to near-infrared light (Kobayashi et al., 2020). For example, an APC that applied rovalpituzumab targeting DLL3 on small cell lung cancer (SCLC) was tested in vitro and in vivo. This study revealed a quick attenuation of tumor and destruction of DLL3-expressing SCLC tumor cells but not in the control non–DLL3-expressing SCLC cells (Isobe et al., 2020). IR700 is a common photosensitizer, a silica-phthalocyanine derivative, that induces immediate cell necrosis after exposure to near-infrared light at 690 nm. To take advantage of combining a photosensitizer and a small cytotoxic molecule, one group designed an antibody-photosensitizer conjugate covered with nanoliposome, which harbored chemotherapy agents inside. This APC-nanoliposome used the antiendothelial growth factor receptor antibody cetuximab, a benzoporphyrin derivative as photosensitizer, and irinotecan as chemotherapy. This construct inhibited tumor growth through a unique three-way mechanism: receptor downregulation, mitochondrial depolarization, and DNA damage (Liang et al., 2020).

There are many other innovations generated by antibody or drug remodeling based on the concept of ADCs. For instance, LDCs use a natural substrate or its derivatives as a navigation system for LDC instead of an antibody (Ge et al., 2020). The ligands include immune checkpoint ligands, such as PD-1 and CTLA-4, tumor-specific growth factor ligands, and metastasis-associated protein ligands. For example, an ADC-like ligand-modified DNA origami nanostructure was reported. The six helical bundles of DNA were modified with a small molecule ligand compound 2-[3-(1,3-dicarboxy propyl)-ureido] pentanedioic acid, which can bind to the prostate-specific membrane antigen. Doxorubicin was loaded in this LDC at high levels. The therapeutic efficacy of this LDC was critically dependent on the ligand numbers (Ge et al., 2020).

Nitric oxide has potential antitumor activity, but lacking a delivery system limits its application. An antibody-nitric oxide conjugate was prepared, containing a CD24-targeting antibody and a nitric oxide–donating diazeniumdiolate anion. This construct efficiently released nitric oxide intracellularly, showing significant antitumor activity in hepatic carcinoma cells in vitro and in vivo (Sun et al., 2019).

Antibody-radioimmuno conjugates (ARCs) can replace small molecule payloads with radioisotope atoms. One ARC containing ^{225}Ac/^{177}Lu with an anti-DLL3

antibody was tested on SCLC and found similar tumor suppression activity on patient-derived xenograft models compared with a PBD-containing ADC (Lakes et al., 2020). Beta radiation has shown activity in patients with refractory prostate cancer. Thus, an alpha-particle emitter thorium-227 ARC targeting prostate-specific membrane antigen was designed and showed strong antitumor efficacy and cancer metastasis inhibition in prostate cancer cell line and patient-derived xenograft models (Hammer et al., 2020). These successful results prompted a phase 1 clinical trial for this ARC.

Small molecules can induce protein degradation via ubiquitin ligases. This technology is called proteolysis-targeting chimeras, and the small molecules are called "degraders." The degraders can efficiently degrade a wide range of biologically important proteins including estrogen receptor, androgen receptor, bromodomain and extraterminal proteins, and various kinases. Still, they are not tissue specific (Maneiro et al., 2020). Therefore, they have therapeutic potential when conjugated to a target delivery tool, like an antibody, to form an antibody-degrader conjugate and have a different mechanism than the traditional small-molecule inhibitors and cytotoxins (Maneiro et al., 2020). For example, Pillow et al. (2020) generated an antibody-degrader conjugate consisting of a picomolar cell potency protein degrader (GNE-987) targeting bromodomain and extraterminal proteins and an anti-CLL1 antibody via a linker. This antibody-degrader conjugate induced antigen-specific tumor regression in vivo. Two distinct ER-α degraders, three independent linkers, and a HER2-targeting antibody were used to form antibody-degrader conjugates and tested their potencies (Dragovich et al., 2020). These conjugates successfully internalized into tumor cells, released the degraders, and showed near-complete degradation of ER-α protein in vitro. In a following in vivo study, several antibody-degrader conjugates with different chimeric BRD4 degrader structures were tested in HL-60 xenograft model mice. They showed delayed tumor growth and even tumor regression with a single 3 mg/kg dose (Dragovich et al., 2021).

B. Drug Conjugates Targeting Cancers with Low Extracellular pH

Apart from delivery based on antibody-antigen binding, other tumor characteristics can be used for drug conjugate delivery, such as those that take advantage of the low pH environment found in tumor tissues. pH(low) insertion peptide (pHLIP) is reported as a pH-dependent delivery system that can accumulate in low-pH tissues and insert into tumor cell membranes. It forms a transmembrane helix and translocates the payload into tumor cells (shown in Fig. 7) (Reshetnyak et al., 2007; Svoronos et al., 2020). pHLIP has been explored successfully for targeted delivery of various drugs ranging from small molecules to macromolecules. pHLIP has been

Fig. 7. Schematic diagram of the mechanism of pHLIP-drug conjugate (pHDC). In a neutral environment (normal-cell extracellular matrix), pHDCs can circulate on the extracellular matrix or cling to the cell membrane and finally achieve a dynamic balance. However, in an acidic environment (tumor-cell extracellular matrix), pHDCs penetrate the cell membrane. The section conjugated with the payload is exposed to the intracellular cytoplasm. The linker can sense the environment difference and break to release the payload. Such linkers include disulfide linkers, which can be cleaved by intracellular reduced glutathione.
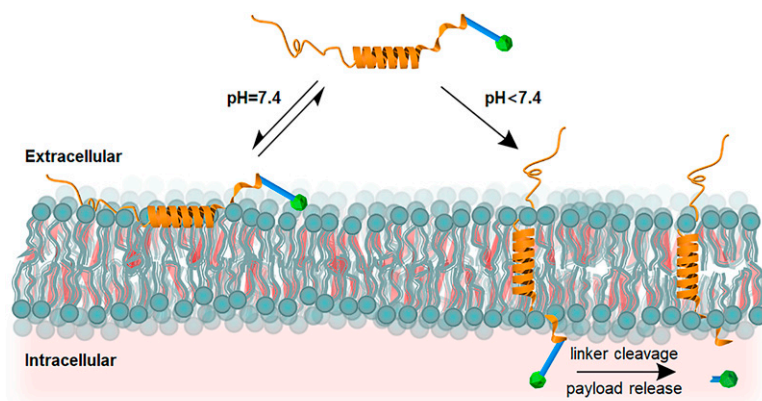
established to deliver antisense nucleic acid analogs for lymphoma therapy in animal studies (Cheng et al., 2015a). Similarly, a small molecule drug conjugate (pHLIP-MMAE) was tested in mouse models. It exhibited pH- and concentration-dependent killing and increased survival time (Burns et al., 2017). In a recent study, the pHLIP-exatecan conjugate was used to selectively inhibit the tumor in vivo. It showed a synergistic antitumor effect with a poly adenosine diphosphate ribose polymerase inhibitor in multiple in vivo tumor models (Gayle et al., 2021). Because nearly all solid tumors have an acidic extracellular environment due to continuing acidosis, the pHLIP-drug conjugate can be applied as a broad-spectrum antitumor reagent.

GALA, another fusogenic, pH-sensitive peptide, is a 30-mer synthetic peptide (composed of glutamic acid-alanine-leucine-alanine repeats with tryptophan and histidine as spectroscopic probes). When the pH changes from 7.0 to 5.0, the peptide transitions from a random, water-soluble coil to an α-helix (Li et al., 2004). Unlike pHLIP, it has been observed that GALA monomers form pores. Approximately 10 GALA monomers form a transmembrane pore, and these pores destabilize the lipid bilayer of the endosome (Li et al., 2004; Wiedman et al., 2017). Therefore, it has been used mainly to aid in the endosomal escape of cargo, such as DNA and small interfering RNA (Li et al., 2004; Sakurai et al., 2009). There are only a few studies on GALA-drug conjugates. In one recent study, GALA was fused to an anti-CD38 nanobody and MMAE. In LP-1 and HEK293T cells, the conjugate was more effective than the anti–CD38-MMAE conjugate without GALA (Chen et al., 2021). In another study, GALA was fused to OKT9, an antitransferrin receptor antibody, to determine the effect of GALA on DAR and activity. They found that the number of GALA per OKT9 antibody was the most significant factor affecting the membrane lytic property. A 1:1 ratio had no effect on the leakage from a lipid bilayer, and 2 to 3 GALA per antibody had the most

significant effect. Moreover, the conjugates were less active than the unconjugated GALA. This was attributed to a decreased lipid partitioning and surface aggregation (Kuehne and Murphy, 2001). GALA sequence can also be changed to modify the charge or improve the endosomal escape property of GALA (Offerman et al., 2014; Boisguérin et al., 2021).

Cell-penetrating peptides consist of 8–30 amino acids that can transfer payload across the cell membrane, but they lack specificity for a cell type. In this regard, activatable cell-penetrating peptides have been developed with triggers such as pH (Dinca et al., 2016). One strategy for producing activatable cell-penetrating peptides is direct conjugation to a payload without an acid-labile bond. In this way, the cell-penetrating peptide would protonate upon exposure to an acidic medium. Dinca et al. (2016) synthesized a cell-penetrating peptide with leucine-histidine repeats that formed dimers through two disulfide bonds conferred by two cysteine residues. Paclitaxel was conjugated to the cell-penetrating peptide for the treatment of TNBC. The cell-penetrating peptide showed a higher α-helicity at the lower pH of 6.0 due to the protonation of the histidine residue at low pH. The disulfide bond also affected this helicity. An in vitro study on MDA-MB-231 cells showed increased pH-dependent uptake compared with a similar cell-penetrating peptide with leucine-lysine repeats, which does not show pH-dependent cationic properties. An in vivo study on a xenograft mouse model showed prolonged circulation and improved tumor growth inhibition with about a tenth of the typical animal doses used for paclitaxel (Nam et al., 2021). In another study, Zhang et al. (2019c) designed a pH-activatable cell-penetrating peptide (LHHLLHHLHHLLHH-NH$_2$) and compared its uptake and cytotoxicity with a nonactivatable cell-penetrating peptide (LKKLLKLLKKLLKL-NH$_2$). In MDA-MB-231 cells, the former showed higher α-helicity and increased cellular uptake at pH 6.0 compared with pH 7.4, whereas the latter showed a similar uptake for both pH values. When conjugated to camptothecin, the

pH-activatable cell-penetrating peptide showed a pH-dependent effect on HeLa and MDA-MB-231 cells as compared with the regular cell-penetrating peptide and free camptothecin. Cell-penetrating peptides and their payloads can also be conjugated through an acid-labile linker. Cheng et al. (2015b) conjugated a cell-penetrating peptide ($CR_8G_3PK_6$) to both a 2,3-dimethylmaleic anhydride and doxorubicin. The 2,3-dimethylmaleic anhydride was designed as a shielding group, blocking the penetrating ability by intramolecular electrostatic attraction. When exposed to a pH of 6.8 in vitro, the shielding group was hydrolyzed and reversed the charge of the cell-penetrating peptide. This increased uptake in HeLa and COS7 cells compared with a control cell-penetrating peptide with a succinic anhydride instead of 2,3-dimethylmaleic anhydride. The shielded peptide also reduced the tumor volume in vivo compared with the control peptide in mice xenografted with H22 hepatic cancer cells. Although outside the scope of this review, it is worth mentioning that the shielding strategy has also been used without conjugating the cell-penetrating peptide to a drug. Instead, micelles or liposomes would be coated with a cell-penetrating peptide shielded by a polyanionic group or PEG through an acid-sensitive linker (Xiang et al., 2017; de Jong et al., 2020).

Other conjugate systems that can exploit the acidic extracellular pH of the cancer cells are pH-responsive polymer-drug conjugates using an acid-labile bond. Typical acid-labile bonds used are hydrazone, acetal, and cis-acotinyl, although oxime bonds and linkers with Schiff-base, β-thiopropionate, or substituted trityl can be used (He et al., 2013; Pang et al., 2016; Rao et al., 2018). Drug molecules conjugated to a polymer are considered prodrugs and are usually inactive. This would reduce the adverse effects, provided that the drug is released efficiently at its site of action (Schmaljohann, 2006).

The pH-sensitive polymers used for conjugation are linear, crosslinked, dendritic, and inorganic polymers. N-(2-Hydroxypropyl)methacrylamide and PEG are the most common linear polymers used for pH-sensitive delivery. Conjugates of N-(2-Hydroxypropyl)methacrylamide have shown comparable cytotoxicity relative to the free drug and even higher cytotoxicity in some cases. These conjugates have shown prolonged circulation and increased tumor retention and inhibition in vivo. PEG, which is usually modified with other polymers, has amphiphilic properties, and can self-assemble into micelles (Pang et al., 2016). Recently, Zhou et al. (2020) developed a pH-sensitive, poly(2-ethyl-2-oxazoline)-poly(D,L-lactide) micelle for codelivery of doxorubicin and curcumin. Curcumin was conjugated to the polymer with an acetal bond whereas doxorubicin was conjugated with a benzoic imine bond. The micelles showed synergistic growth and meta-stasis inhibition in MDA-MB-231 cells and reduced adverse effects compared with the micelles, which were only physically loaded with both drugs. One issue with these self-assembled micelles is a premature drug release during systemic circulation. Core-crosslinked micelles can be used to mitigate this and increase their colloidal stability. Dendritic polymers have also been developed. They have favorable characteristics such as low polydispersity index, controllable size, and many tunable terminal groups for conjugation. However, due to the toxicity issues of dendrimers, they are PEGylated. PEGylated dendrimer-drug conjugates have shown strong pH-responsive drug release with no associated toxicities. Lastly, inorganic polymers including single-walled carbon nanotubes, gold nanoparticles, and mesoporous silica nanoparticles have been studied both as a therapeutic and diagnostic system (Pang et al., 2016).

pH-sensitive polymers can also help with the intracellular delivery of the conjugates leading to endosomal or lysosomal escape (Schmaljohann, 2006; He et al., 2013). In the study of Du et al. (2011), a dual pH-sensitive polymer drug conjugate was synthesized. To achieve this, the cysteamine modified diblock copolymer monomethoxyl poly(ethylene glycol)-b-poly-(allyl ethylene phosphate) was conjugated to doxorubicin via a hydrazone bond and then to 2,3-dimethylmaleic anhydride. These constructs self-assembled into negatively charged nanoparticles in water. Upon being exposed to a pH of 6.8, they reversed their charge from negative to positive, which helped with their endocytosis. These dual pH-sensitive conjugates showed improved uptake compared to a control conjugate. In MDA-MB-231 cells, it was observed that the hydrazone bond would break to help with the intracellular release of the drug from the acidic endosomes as compared to control conjugates. Instead of conjugation, pH-responsive polymers such as micelles, liposomes, and dendrimers can also encapsulate the drugs and release them once in the acidic extracellular space or once inside the cells (He et al., 2013; Kang et al., 2014).

1. Nanotechnology. Targeted drug delivery with nanoparticles has been explored for decades with the promise of increasing the efficacy and/or reducing the toxicity of their cargo by enhancing their solubility, targeting, and releasing cargo by external stimuli (Hua et al., 2018). Nanoparticles are a formulation-based approach to drug delivery. Nanoparticle technology can generate antibody-drug conjugate nanoparticles (ADCNs) (Fig. 8) (Johnston and Scott, 2018).

a. Antibody-drug conjugate nanoparticles and their advantages. ADCNs are composed of a nanoparticle drug delivery system conjugated to an antibody. In ADCNs, the cargo is not directly conjugated to the antibody as it is in ADCs. Conjugating nanoparticles with antibodies promotes active targeting of the
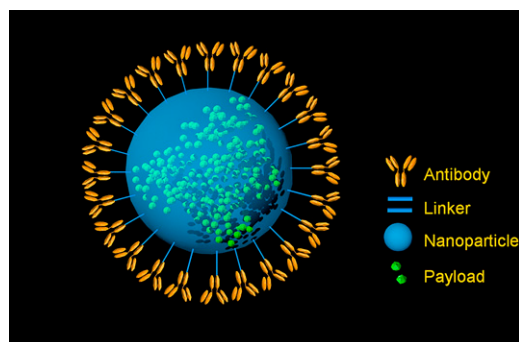
Fig. 8. Diagram of ADCNs. These conjugates can self-assemble into nanoparticles via hydrophobicity and hydrophilicity interactions of the payload, linker, and antibody. ADCNs can carry plenty of payloads inside the nanoparticle (very high DAR) and display antibodies on their surface for targeted delivery.

nanoparticle, delivering the cargo selectively by binding to overexpressed antigens on the cancer cells in the tumor microenvironment. Compared with passively targeted nanoparticles that lack a targeting ligand, such active targeting of nanoparticles with antibodies avoids multidrug resistance transporters. This is due to the ADCN being internalized through receptor-mediated endocytosis (Cardoso et al., 2012; Attia et al., 2019; Marques et al., 2020). ADCNs can alleviate some limitations of ADCs. Traditional ADCs require precise chemical synthesis, including multiple-product-losing reaction steps (Frigerio and Kyle, 2017). However, the preparation of ADCNs mainly relies on self-assembly. Within tumor cells, ADCs with cleavable linkers often release charged linker-containing payloads, directly affecting their potency (Bargh et al., 2019). In contrast, ADCNs can release nonconjugated payloads, ensuring their efficacy. Compared with ADCs, drugs with lower potency can be used in ADCNs, which provides the option to select a wider variety of cargos. Not using highly potent cytotoxic drugs also has the benefit of reducing potential adverse effects. Moreover, in ADCNs, more drugs can be encapsulated inside the nanoparticle component, and therefore a higher DAR can be achieved, whereas for ADCs, the DAR needs to be carefully controlled

(Tang et al., 2017). On average, ADCs have DARs ranging from 1 to 8, whereas the DAR of ADCNs can reach to as high as 100 (Debbage, 2009). In addition, it is convenient for ADCNs to carry many different antibodies that can recognize and attack tumor cells with different target antigens concurrently. For instance, a hydrophilic drug can be loaded inside a liposome and a hydrophobic one can be loaded between its hydrophobic bilayer (Fay and Scott, 2011). This is also a promising way to target heterogeneous tumor microenvironments and drug resistant tumors (Johnston and Scott, 2018). Finally, because of having more paratopes, some ADCNs can induce a higher order of receptor clustering (the formation of localized groups or clusters of receptors previously distributed on the cell membrane), which could promote more robust signaling activation compared with the respective free antibody or ADC (Schmid et al., 2014). These differences are summarized in Table 3.

b. Conjugation strategies. The conjugation of the antibody to the nanoparticle can be achieved by two general strategies: noncovalent interactions or covalent bonds. There is no single strategy that would be suitable for all conjugation needs since the nanoparticles and the antibodies both have unique features to consider before conjugation. Nanoparticles differ based on their structure and design, material, size, shape, surface area, type of functional groups, and colloidal stability. Antibodies are unique in their chemical composition, size, available residues for conjugation on their Fc region, and 3D structure (Sapsford et al., 2013; Parracino et al., 2019).

The covalent approach is more commonly used (Friedman et al., 2013; Eloy et al., 2017), but it is challenging in comparison with nonspecific adsorption methods (Kadkhoda et al., 2021). It involves the chemical coupling of the nanoparticle and antibody. Hence, both nanoparticles as well as antibodies need to be functionalized to generate enough and high-quality ADCNs (Friedman et al., 2013; Parracino et al., 2019). The common chemistries used for conjugating the nanoparticles with ligands including antibodies are: (1) reaction of carbonyl-reactive groups such as hydrazide or alkoxyamines on nanoparticles

TABLE 3
Comparison of ADC and ADCN features

	ADC	ADCN
Antibody requirement	A single antibody is used to bind one target	Multiple antibodies can be added to target heterogeneous and/or drug resistant tumors
Linker influence	Linkers affect payload potency and can cause side effects, especially with cleavable linkers	Target binding and payload are not affected by linkers
Payload requirement	Highly potent drugs are needed	Drugs with lower potency can be used
DAR	Has a limited DAR range (1–8)	Higher amount of drug can be encapsulated (can reach >100)
Single/multiple-type payload	One type of payload is primarily used	Multiple types of payloads can be loaded
Receptor clustering	Regular receptor clustering (in classic, monospecific antibodies)	Potential for enhanced receptor clustering
Preparation	Precise chemical synthesis with multiple reaction steps	Mainly relies on self-assembly

with an aldehyde tag added on the antibody to form a hydrazone linkage, (2) reaction of amine-reactive groups such as carboxylate or imidoester on nanoparticles with the primary amine of antibody to form amide bond or amidine bond, respectively (the primary amine can be part of the natural backbone or be an engineered residue whereas the carboxylate residue of the nanoparticle is activated by using a carbodiimide crosslinker), (3) Reaction of sulfhydryl-reactive groups such as maleimide and haloacetyls with a sulfhydryl group of an antibody to form a thioether linkage, (4) reaction of sulfhydryl groups on both the nanoparticle and the antibody to form a disulfide linkage, and (5) reaction of azide-alkyne cycloaddition click chemistry to produce a triazole ring linkage or the biorthogonal azide-phosphine reaction to form an amide bond (Friedman et al., 2013).

Amino acids with ionizable side chains, including aspartic acid, glutamic acid, lysine, arginine, cysteine, histidine, and tyrosine, are the most commonly used for nanoparticle conjugation to antibodies (Hermanson, 2011). The limitation of the aforementioned technology is that the antibody might be conjugated in different orientations due to the presence of similar residues on the antibody surface (Friedman et al., 2013; Oliveira et al., 2019; Parracino et al., 2019). Correct orientation is paramount for the biologic activity of the antibody and its stability under denaturing conditions such as pH and temperature (Parracino et al., 2019). The number of the attached antibodies might not be desirable in some cases. Using noncanonical amino acids and biorthogonal reactions can resolve some of the aforementioned limitations (Friedman et al., 2013). A combination of a two-step adsorption followed by covalent conjugation has been used to modulate the orientation of antibodies on magnetic nanoparticles (Puertas et al., 2011).

Noncovalent strategies include nonspecific adsorption and affinity-based interactions (Parracino et al., 2019). Passive adsorption can result from van der Waals interactions, hydrogen bonding, hydrophobic interactions, and electrostatic attraction (Goossens et al., 2017). These methods are cheaper, faster, and easier in comparison with covalent approach (Goossens et al., 2017; Kadkhoda et al., 2021). In addition, there is no need for chemical modification of the nanoparticles and antibodies. However, the major limitation is that these noncovalent adsorption systems are fundamentally weaker and reversible (Parracino et al., 2019). The interaction of the nanoparticle and antibody might easily be competitively displaced by other molecules in an in vivo environment (Cardoso et al., 2012). Also, some of the passive adsorption techniques, such as ionic adsorption, exhibit poor reproducibility, uncontrolled orientation, and low stability at various pH conditions (Oliveira et al., 2019). There is also the need for a lot of antibody, which contributes to cost of manufacturing (Marques et al., 2020).

The affinity-based interaction is simple, commonly used, and relies on the interaction between avidin and biotin to conjugate the nanoparticle and antibody. Biotin and biotin-binding protein (avidin or its derivatives) have the strongest natural noncovalent bond known with a Kd of 10^{-14} to 10^{-16} (Friedman et al., 2013; Parracino et al., 2019). These interactions are very stable and resistant to harsh chemical conditions and high temperatures (Sperling and Parak, 2010). Usually, biotin-binding proteins are either adsorbed directly on the nanoparticle surface or coupled by covalent binding, and the antibodies must be biotinylated (Parracino et al., 2019). The reverse can also be performed by biotinylating the nanoparticle and conjugating the antibody with avidin (Palanca-Wessels et al., 2016). Avidin is a tetrameric molecule, and thus one limitation of this method is that the antibody stoichiometry is challenging to control. In this regard, the dimeric form (with a comparable affinity to the tetramer) and monomeric form (with much lower affinity for biotin) have been explored (Meir et al., 2009; Parracino et al., 2019).

c. Targets used with antibody-drug conjugate nanoparticles. Similar to ADCs, the research on ADCNs has mainly focused on targeting tumors using a cytotoxic agent (Fay and Scott, 2011). Various receptors overexpressed in different cancers have been targeted with these conjugates. HER2 is the most explored target. Other targets include vascular endothelial growth factor, prostate-specific membrane antigen, and transferrin receptor for targeting breast cancer, lung cancer, pancreatic cancer, lung cancer, melanoma, and brain tumors (Arruebo et al., 2009; Fay and Scott, 2011; Johnston and Scott, 2018).

There are also reports of using antibody-guided nanoparticles, which deliver a gene instead of a cytotoxic agent (Chiu et al., 2004; Hayes et al., 2006; Mann and Kullberg, 2016). Chiu et al. (2004) fabricated plasmid DNA-linear polyethyleneimine polyplexes that were conjugated to the trastuzumab anti-HER2 antibody for tumor gene therapy. They evaluated the transfection efficiency of these constructs in HER2 overexpressing Sk-Br-3 and HER2 low-expressing MDA-MB-231 breast cancer cell lines. They observed anti-HER2–mediated transfection of the targeted polyplexes in Sk-Br-3 cells, whereas in the low expressing cells, they found no significant difference between the targeted and nontargeted polyplexes. Moreover, the transfection efficiency of the polyplexes was dependent on the trastuzumab:polyethyleneimine ratio. Mann et al. (2016) developed a gene delivery system for targeting HER2-overexpressing isogeneic MCF7 and MCF7/Her18 breast cancer cells. This system consisted of a PEGylated polylysine-DNA complex covalently linked to trastuzumab and listeriolysin O via disulfide bond. Listeriolysin O is a pore-forming protein that ensures DNA transport from the endosomes

into the cytoplasm. This delivery system resulted in a 30-fold greater expression of luciferase activity in these cells. Their generic design allows for utilizing another plasmid DNA and antibodies. Hayes et al. (2006) developed an immune-targeted cationic lipid-plasmid DNA, PEGylated nanoparticle platform. PEG is used in nanoparticle drug delivery systems to improve pharmacokinetic properties of the particles, but it also hinders uptake to the targeted cells. To overcome the PEG-related inhibition of particle-cell association, they incorporated anti-HER2 scFvs in the nanoparticles, which showed high transfection activity in SK-BR-3 HER2 overexpressing breast cancer cell lines.

Gene delivery of tumor-suppressing genes using ADCNs has also been tested in clinics (van der Meel et al., 2013). Palanca-Wessels et al. (2016) studied the efficacy of small interfering RNA-polymeric micelle complexes linked to streptavidin-conjugated trastuzumab for breast and ovarian cancer treatment. HER2-overexpressing SKOV3 ovarian cells demonstrated an 80% reduction of glyceraldehyde-3-phosphate dehydrogenase expression for at least 96 hours. A similar effect was observed in Sk-Br-3 cells. In ovarian cancer xenograft model mice, a 70% suppression was observed.

ADCNs have also been used as contrast agents during in vivo imaging of tumors such as breast cancer, lung adenocarcinoma, and pancreas cancer. Although they are mainly used for improving the contrast of magnetic resonance imaging and computerized tomography, near-infrared and fluorescence optical imaging platforms are also being explored (Farahavar et al., 2019; Kadkhoda et al., 2021). Although the research on ADCNs primarily focuses on oncology, a few other applications have also been reported. For instance, Nanaware-Kharade et al. (2012) designed and synthesized a single-chain antimethamphetamine antibody fragment conjugated to a PEG-modified dendrimer delivery system for treating methamphetamine addiction. They then characterized this system for its size, purity, and methamphetamine-binding affinity. These constructs had an identical affinity for methamphetamine as the unconjugated antibody in saturation binding assays, suggesting that the conjugation process had no adverse effect on methamphetamine binding properties. Moura et al. (2014) prepared and characterized methotrexate-loaded, superparamagnetic iron oxide nanoparticle-containing poly lactic-co-glycolic acid nanoparticles conjugated with anti-CD64 with the potential for simultaneous imaging and targeting of rheumatoid arthritis. Arias et al. (2015) fabricated and studied the efficacy of nanobody-conjugated PEGylated poly lactic-co-glycolic acid nanoparticles loaded with pentamidine to treat African trypanosomiasis. In vitro assay of the constructs showed a sevenfold decrease in IC$_{50}$ compared with the free drug. In vivo evaluation of the formulation in a murine model of this disease cured all infected mice at a 10-fold lower dose relative to the free drug.

d. Nanoparticles and antibodies used for antibody-drug conjugate nanoparticle development. A wide range of nanoparticles has been used for developing ADCNs (Johnston and Scott, 2018). The most notable ones include polymeric nanoparticles, especially poly lactic-co-glycolic acid, but also polystyrene, polylactide, liposomes ("immunoliposomes"), and inorganic nanoparticles such as silica nanoparticles, iron oxide nanoparticles, and gold nanoparticles (Friedman et al., 2013). The type of cargo and the intended use mostly decide the choice of nanoparticle formulation (Johnston and Scott, 2018). For example, magnetic nanoparticles are particularly suited for theranostic applications as contrast-enhancing agents in magnetic resonance imaging and computerized tomography (Kadkhoda et al., 2021). Most of the antibodies used for ADCN are monoclonal and of murine origin (Friedman et al., 2013). Complete antibody molecules and their fragments have been used for ADCNs production (Mann and Kullberg, 2016; Johnston and Scott, 2018). Smaller antibody fragments have the potential advantages of diffusing and accumulating more rapidly into the tumor, having a higher binding affinity with their receptor, and being easier to produce (Xenaki et al., 2017). Cargos used with ADCN design mainly include cytotoxic drugs such as doxorubicin, docetaxel, and paclitaxel (Farahavar et al., 2019; Juan et al., 2020; Kadkhoda et al., 2021).

C. Visualization of Cancer Therapy

Cancer therapy visualization offers a more direct judgment on drug treatment compared with biochemical tests. During the development of an ADC molecule, imaging techniques are initially used to examine the ability of the antibody portion to target its antigen and subsequent response to treatment and its fate once internalized inside the cells (Cohen et al., 2014; Azhdarinia et al., 2018). Visualization is also a preferable method, with more possibilities for assessing in vivo drug conjugate distribution, behavior, efficacy, and mechanism. In addition, visualization of the ADCs will help with standardizing their dosing schedule and performing "before" and "after" evaluations of the tumor (Lyons et al., 2021).

The first method in visualization of cancer therapy is target visualization through transfection of a fluorescent protein gene into cancer cell lines, such as green fluorescent protein, or a luminescent protein, such as firefly luciferase. When these cancer cell lines are used as a tumor model, the fluorescent or luminescent proteins show the in vivo location of the tumor mass and therapeutic effect. For example, a tumor cell line expressing green fluorescent microtubule tracking protein and a corresponding mouse model were established. They are suitable for visualizing tumor cell invasion, proliferation and

metastasis, as well as microtubule dynamics in response to drug conjugates that damage the microtubule (Gonda et al., 2020). Bioluminescence imaging is commonly used to measure the cytotoxicity of the ADC in vitro and in vivo due to its sensitivity, speed, and affordability. In this method, cells are transduced with luciferase enzyme, which depends on the ATP of the live cells as the substrate, to produce light. This allows for monitoring the tumor before it is visually apparent (Lyons et al., 2021).

Drug conjugates can be directly labeled with a fluorescent probe or a radioactive atom, such as fluorescein isothiocyanate or ^{89}Zr, both of which allow their connected ADC to be detectable and imageable. These type of drug conjugates are antibody-dye conjugates or antibody-drug-dye conjugates. For instance, a dual-labeled ADC, such as trastuzumab-MMAE-^{89}Zr, can be used for tumor therapy as well as visualizing ADC delivery. The choice of imaging technique depends on the purpose of the experiment (Lyons et al., 2021). Among the imaging techniques used to study the ADC biodistribution, tagging the unconjugated antibody with a radioisotope is the most common and is also known as antibody-based positron emission tomography (PET). This method is commonly used in the preclinical and clinical settings for identifying tumors with high expression levels of a target. The utility of this technique can be expanded to the ADCs as well (Azhdarinia et al., 2018; Carmon and Azhdarinia, 2018; Lyons et al., 2021). The antibody-radionuclide conjugate would help as a surrogate to predict the final ADC performance in vivo (Carmon and Azhdarinia, 2018). PET imaging appears to be the most sensitive way to track ADC biodistribution (Lyons et al., 2021).

In antibody-based PET, various radionuclides such as copper-64, zirconium-89 (^{89}Zr), and iodine-131, which have different half-lives, label the antibody (Azhdarinia et al., 2018). The radionuclide itself can also be considered as a payload, killing nearby cells by radiation (Altunay et al., 2021). ^{89}Zr is commonly used due to its appropriate half-life (3.3 days). This improves the tumor visualization because it would allow the nontarget tissues to be cleared of the tracer and also facilitate the imaging process for patients (Azhdarinia et al., 2018). Antibody fragments, which themselves have a short half-life, can be used with radioisotopes that have a shorter half-life than ^{89}Zr. Antibody fragments have the disadvantage of being eliminated faster, thus reducing tumor uptake (Ilovich et al., 2015). Also, a retrospective analysis applied antibody-dye conjugate to test ADC delivery. It was found that coadministering with the parent antibody improved intratumoral ADC penetration without increasing uptake by healthy tissue. This was demonstrated by treating patients with antibody-dye conjugate and antibody and then surgically resecting

the tumor for macroscopic and microscopic imaging (Lu et al., 2020).

Photodynamic therapy can also be used for imaging. When conjugated to an antibody and triggered by a specific wavelength light to generate singlet oxygen and reactive oxygen species, these constructs would induce cancer cell apoptosis and necrosis (Deken et al., 2020). Deken et al. (2020) designed a nanobody photodynamic therapy conjugate consisting of a HER2-targeted nanobody and a photosensitizer. It targeted HER2-overexpressing, trastuzumab-resistant cells with low, nanomolar LD_{50} values. Examination by quantitative fluorescence spectroscopy showed accumulation into tumor tissue 2 hours postinjection. It induced significant tumor regression of high-HER2–expressing tumors while delaying the growth of low-HER2–expressing tumors with a single treatment session.

Only a few reports directly label the ADC with a radionuclide (Adumeau et al., 2018). In one experiment, Cohen et al. (2014) monitored the conjugation process and purification of the ADCs by dual labeling both the antibody and the cargo. This technique allowed the detailed examination of in vitro and in vivo stability of the ADC. The stability of the conjugate is critical to ensure the toxic cargo is not released prior to reaching its target site. In another experiment on mice with human prostate cancer explant, Boswell et al. (2012) studied the effect of predosing with an unlabeled and unconjugated antitomoregulin (anti-TENB2) on the biodistribution of the ^{111}In-labeled anti–TENB2-MMAE. They reported that the systemic exposure of the labeled ADC increased significantly, and the intestinal, hepatic, and splenic uptake decreased without affecting tumor accumulation. More recently, a study with a higher tracer dose at 1 mg/kg and a low TENB2-expressing model was performed, but no efficacy was observed with this dose (Boswell et al., 2019). Fluorescent probes can also be used, but they are not as useful for deep tissue imaging (Yasunaga et al., 2017). In a recent study, Xiao et al. (2021) developed and evaluated the in vivo and in vitro effect of a bifunctional, theranostic anti-HER2 antibody conjugated with both MMAE and 7-amino-3-hydroxyethyl-coumarin, a dipeptide linker with on-off fluorescent properties. Other techniques, such as optoacoustic imaging, have been used for preclinical deep tissue imaging with a good compromise of sensitivity and resolution, although it cannot provide subcellular images (Lyons et al., 2021).

V. Barriers to Clinical Translation

A. Barriers in Preclinical Studies

1. Drug Antibody Ratio. DAR has been an essential issue to drug conjugates since the first ADC drug, Mylotarg, came into the market. It directly determines the quality of manufacture, the therapeutic effects, and

adverse reactions. Although on average, the DAR of Mylotarg is 2.5, large percentages of the antibodies are not bound to any payload, whereas the rest have a DAR of 4 to 5 (Joubert et al., 2020). The DAR can alter the pharmacokinetic properties of an ADC. A higher DAR causes more rapid clearance and increases the risk of immunogenicity (Mckertish and Kayser, 2021). Although reducing the DAR reduces the efficacy of an ADC, a higher DAR can negatively impact the antibody stability (Tang et al., 2017) and increase the risk of toxicity. Therefore, the toxicity and delivery of drug conjugates should be carefully balanced. Another aspect of the DAR to be considered is site-specific conjugation (Donaghy, 2016). This factor impacts antigen binding, stability, and pharmacokinetic properties as well, and can increase the therapeutic index (Yamada and Ito, 2019). If payloads are attached within or close to the antigen recognition regions on the antibody, the targeted delivery will be a problem. Therefore, achieving a consistent DAR, or at least a DAR within a narrow range, together with site-specific conjugation help to manufacture a more uniform and easier-to-characterize product with a more predictable in vivo performance (Tian et al., 2014).

Although ADCNs can reach a high DAR, achieving a consistent DAR or antibody-to-nanoparticle ratio is also important. Site-specific conjugation utilizing unAA and biorthogonal reactions are among strategies for remedying this (Friedman et al., 2013). For example, one group used azide-alkyne cycloaddition and microbial transglutaminase for site-specific conjugation of antibodies to plant virus nanoparticles. They successfully prepared stable and functional antibody-nanoparticles using several antibodies such as trastuzumab. The trastuzumab-nanoparticles can specifically bind to HER2$^+$ human ovarian cancer cells (Park et al., 2020). Another group used a UV photo-crosslinking method for site-specific conjugation of anti–prostate-specific antigen to gold nanoparticles (Mustafaoglu et al., 2017).

2. Drug Resistance.   Another obstacle to drug conjugate success is the occurrence of resistance. Tumors may generate resistance to ADC treatment, which narrows the therapeutic index of an ADC. Resistance to ADCs can be divided into two types of mechanisms. One resistance mechanism is found at the interface of antibody-antigen binding, such as the antigen downregulation, loss, or mutation preventing ADC binding (Szot et al., 2018; Hafeez et al., 2020). These changes can generate tumor heterogeneity, with some tumor cells lacking particular tumor markers (Boshuizen et al., 2018). For example, as a mechanism of treatment resistance, advanced prostate cancer can histologically transform into small cell neuroendocrine prostate cancer, changing its cell surface antigens (Puca et al., 2019). Situations like this substantially increase the difficulty of treating tumors. However, use of bispecific antibodies, targeting widely expressed antigens, targeting stromal antigens, combining multiple antibodies into the same ADCN, or combined use of various ADCs may be able to solve these problems.

The other resistance mechanism is found at the interface of payload-target binding. This type of resistance is due to the overexpression of drug efflux transporters such as P-glycoprotein to escape payload cytotoxicity. One strategy to reduce recognition by efflux transporters is to reduce the hydrophobicity or increase the charge of the linker and/or payload because these transporters mostly efflux hydrophobic compounds. However, alteration of linker or payload hydrophobicity can also impact formation of ADC aggregates (Collins et al., 2019; Hafeez et al., 2020), which can increase the risk of immunologic side effects (Mckertish and Kayser, 2021). Incorporating this strategy also reduces the bystander effect, which causes its own problems (Collins et al., 2019; Hafeez et al., 2020). Without a bystander effect, for instance in trastuzumab emtansine with a positively charged payload, the subpopulation of cells with low HER2 receptor expression could increase, eventually leading to an acquired resistance (Collins et al., 2019).

Payload-target binding can also be impacted by changes to intracellular trafficking that block ADC internalization, reduced lysosomal proteolytic activity that block payload release, and mutations to the target that reduce binding (Collins et al., 2019). Resistance does not likely arise from a single event; rather, tumor cells can constantly adapt to continued selective pressure by modifying the various steps necessary for the payload to exert its cytotoxic effect (Collins et al., 2019). To resolve problems at the interface of payload-target binding, light-cleavable ADC, ARC and click-to-release ADC may show better therapeutic efficacy. Moreover, long-term treatment of a single tumor target will increase the probability of drug resistance to this target and is a common cause of treatment failure (Vasan et al., 2019). To resolve this problem, multiple payloads on one ADC, dual-targeting warheads, or multiple payloads contained within an ADCN may be promising.

3. Other Preclinical Problems.   Tumor antigen expression should be fully considered before the drug conjugates move forward in development. Expression of a target antigen in normal tissues could cause toxicity, or binding with a low affinity could present a lower therapeutic effect, although these can be screened in a preclinical setting. Low-affinity or nonspecific binding might also prompt antibody-mediated toxicity (Donaghy, 2016). In addition, the accessibility of tumor antigens is also a problem. Exclusive tumor antigen expression primarily has been observed for hematologic antigens. Therefore, most of the approved ADCs or candidates in trials are targeted for these malignancies rather than solid tumors (Criscitiello et al., 2021).

Tissue penetration of drug conjugates, especially for solid tumors, is another issue they are facing for clinical translation. Increasing the construct stability and half-life of the ADC would help improve their accumulation (Tsumura et al., 2018). Once they reach the tumor, drug conjugates will distribute unevenly due to several factors such as abnormal vasculature and the binding site barrier (Bordeau et al., 2022). This barrier stems from antibodies binding to antigens that are located close to capillaries, with lower amounts of the antibodies then available to reach more distant binding sites. To overcome the binding barrier, coadministration with free antibody has been used successfully, although it seems this approach might only work for tumors with very high expression levels of an antigen (Lu et al., 2020; Singh et al., 2020a). Studying antibody biodistribution before and after conjugation, including antibody-dye conjugates, may help better understand tissue penetration (Coats et al., 2019). To overcome solid tumor penetration problems, the aforementioned miniaturized ADC, SMDC, LDC, and drug conjugates targeting cancers with low extracellular pH technologies may have more potential due to their small sizes.

Plasma stability is another important aspect in preclinical studies, especially because human and murine models show differences in metabolism. For example, dipeptide linkers are unstable in rodent models due to the Ces1C hydrolase enzyme. An ideal linker must be stable in the human blood circulation and only cleave once in the site of action (Donaghy, 2016). Linker instability of the first FDA-approved ADC, Mylotarg, caused premature drug release and toxicity, leading to its withdrawal from the market. It was reapproved by modifying the dose and dosing regimen (Joubert et al., 2020).

Finally, a big problem exists in predicting the safety and efficacy of drug conjugates. Correlating the in vivo efficacy and safety of preclinical studies to the clinical trials has proven to be difficult. In several cases, there is a promising preclinical complete response in murine models but lack of such strong effects or occurrence of toxicity in clinical stages. This stems from the fact that the target antigen might not be expressed in the normal tissues of the animal model, which skews the interpretation (Tolcher, 2016).

B. Barriers in Advanced Clinical Stages

A survey of discontinued ADC trials shows two main reasons for the failure of ADCs to move to advanced clinical stages: (1) lack of efficacy and (2) unexpected toxicity. For instance, the phase 3 clinical trial of rovalpituzumab tesirine was terminated because it failed to meet its primary endpoint: improving overall survival in small cell lung cancer (Johnson et al., 2021). In this clinical trial, rovalpituzumab tesirine treatment exhibited an overall survival of 8.5 months, and placebo induced overall survival of 9.8 months. Meanwhile, the rovalpituzumab tesirine treatment group showed adverse events such as pleural effusion (27%), decreased appetite (27%), peripheral edema (26%), photosensitivity reaction (25%), fatigue (25%), nausea (22%), and dyspnea (21%) (Johnson et al., 2021). In another example, although it exhibited excellent activity in a preclinical study, a vadastuximab talirine phase 3 trial for acute myeloid lymphoma was halted due to a higher death rate and fatal infection in the treatment arm than placebo (NCT02785900; NCT02706899).

A limited number of ADCNs have reached the clinical trial stage, and none to our knowledge has yet advanced to phase 3 (Cardoso et al., 2012; Eloy et al., 2017; Hua et al., 2018; Johnston and Scott, 2018). Like ADCs, the success of ADCNs requires a tumor-specific or tumor-associated antigen and a high-affinity antibody targeting that antigen (Juan et al., 2020). However, there are some additional problems that ADCNs may face due to the complexity of the constituents. For instance, nanoparticles often have poorer tumor accumulation than other drugs. An analysis of publications with in vivo data showed that only a median of 0.7% of the administered nanoparticle dose actually reaches the tumor. This can be attributed to the inadequate understanding of tumor-targeting mechanisms of different nanoparticles (Wilhelm et al., 2016). Other challenges included achieving a consistent antibody-to-nanoparticle ratio, correct orientation, and stability during systemic circulation (Friedman et al., 2013). For instance, noncovalent conjugation might not produce a stable conjugate once injected into the peripheral blood. Finally, until the in vivo efficacy of ADCNs is demonstrated, there will not be much interest in their large-scale manufacture, so scale-up can remain difficult (Johnston and Scott, 2018).

C. Unanswered Questions

Beyond the technologies discussed in this review article, there are still many unanswered questions related to drug conjugates that are worth exploring for better drug conjugate design. First of all, for design of optimal drug conjugates, scientists may think about these two questions: (1) how to determine the best targeting agent for a particular solid tumor, because miniaturized antibodies with low molecular masses have better tumor tissue penetration, at the expense of plasma half-life and (2) is it possible to design a "perfect" ADC conjugation technology with high DAR, short conjugating time, high linker plasma stability, and low cost in preparation?

To expand drug conjugates application, three questions need to be addressed: (1) Beyond DNA and microtubules, what other intracellular targets can be targeted by ADC payloads? (2) Can a map be developed to guide the selection of tumor-specific antigens and tumor-associated antigens in specific tumor types

for targeting with drug conjugates? (3) Will tumor stroma–targeted ADC replace the traditional tumor cell internalization-dependent ADC or become the trend of ADC development in the future?

New drug conjugates showed enriched diversity and infinite possibilities; meanwhile, we may ask: (1) Will ADC nanoparticles show better safety and efficacy than traditional ADCs in clinical trials? (2) Among the X-drug conjugates, which will replace ADCs as the mainstream technology of targeted therapies in the future? (3) Can visualization of tumor therapies obtain market approval and higher application value? It will be exciting to see how these preclinical technologies continue to bloom together to enable the clinical application of new drug conjugates.

VI. Conclusions and Perspectives

Nearly 100 years was required for Paul Ehrlich's idea of the "magic bullet" to come true. Every year, several new ADCs are being successfully launched. Scientists are no longer satisfied with the existing ADCs and ADC structures but want to solve the problems faced in ADC marketing, introduce improvements as well as expand the application scope of ADC, and develop new types of drug conjugates. There is a large amount of exciting preclinical research ongoing in the area of drug conjugates. These efforts are likely to yield compounds that enter clinical trials or methods that become standard experimental approaches in the future. Most importantly, they broaden the thinking of ADC design. For antibodies, the highest priority is to solve the problems that prevent high-quality production of natural antibodies, synthetic antibodies, and antibody derivatives (Harel and Benhar, 2012). With advancing antibody technology, ADC antibody selection and application will greatly benefit. In addition, for selection of the optimal antibody form, work on the distribution characteristics, tumor tissue penetration, internalization rate, circulating half-life, and other factors is needed (Birrer et al., 2019). For linkers, more and more studies are focusing on simplifying the steps involved in the conjugation. Past linker conjugation required multistep chemical reactions, with each step leading to product loss and raw material waste. Now, incorporation of unAA, cyclo-synthesis, and other click chemistry reactions cannot only solve the problem of heterogeneity (variable DAR) but also simplify the syntheses. For payloads, most current ones necessarily have high toxicity at the cellular level. Research directions are still relying on new payload scaffolds and discovery of alternative intracellular targets (Buecheler et al., 2020). Identification of new tumor surface targets and studies of their biologic functions are also important to benefit ADC target selection and increase knowledge of tumor biology (Schumacher et al., 2019). Advances in these areas is being enabled by the continued development of analytic technology used for characterization of ADCs, focusing on the rapid and intelligent analysis of DAR, plasma stability, and ADC metabolites. Many detection methods have been developed, but additional studies are needed to broaden the scope of their application (Yan et al., 2020).

In recent years, the overlap of ADCs with other research fields has given birth to some interesting new directions like ADC nanoparticles, X-drug conjugates, and visualization of tumor therapy. ADC nanoparticles have advantages with respect to their ability to be loaded with high amounts of payload and their ability to carry multiple antibodies or multiple payloads (Cardoso et al., 2012). In the future, novel nanoparticle and polymer ADCs will continue to appear. X-drug conjugates have advantages with respect to their smaller size and potential for better tumor penetration. However, their clinical transformation is still challenging due to their novel design with limited research. Visualization tumor therapy has advantages with respect to guiding personalized therapies through the combination of antibody-dye conjugate and ADC (Gonda et al., 2020). The research is limited at present, but it is with great hope to develop more powerful tools in this area in the future. Together, drug conjugates are promising tumor treatment approaches with more and more research on their properties occurring with every new idea. As a result, branches of drug conjugate research are increasing yearly, which together all provide the foundation for improving tumor treatment options (Tolcher, 2020).

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