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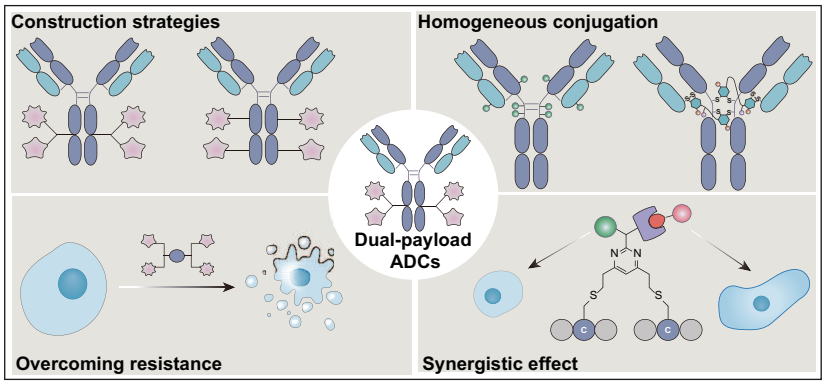

Dual-payload Antibody–drug Conjugates: Taking a Dual Shot

Junjie Tao[1#], Yilin Gu[1#], Wei Zhou[3]*, Yuxi Wang[1, 2]*

[1]Targeted Tracer Research and Development Laboratory, Institute of Respiratory Health, Frontiers Science Center for Disease-related Molecular Network, National Clinical Research Center for Geriatrics, West China Hospital, Sichuan University, Chengdu, 610041, Sichuan, China

[2]Frontiers Medical Center, Tianfu Jincheng Laboratory, Chengdu, 610212, Sichuan, China

[3]Mabwell (Shanghai) Bioscience Co., Ltd, Shanghai 201210, China

[#]These authors contributed equally to this work.

*Corresponding author.

E-mail address: yuxiwang@scu.edu.cn (Y. Wang); wei.zhou@mabwell.com (W.Zhou)

Abstract

Antibody-drug conjugates (ADCs) enable the precise delivery of cytotoxic agents by conjugating small-molecule drugs with monoclonal antibodies (mAbs). Over recent decades, ADCs have demonstrated substantial clinical efficacy. However, conventional ADCs often encounter various clinical challenges, including suboptimal efficacy, significant adverse effects, and the development of drug resistance, limiting their broader clinical application. Encouragingly, a next-generation approach—dual-payload ADCs—has emerged as a pioneering strategy to address these challenges. Dual-payload ADCs are characterized by the incorporation of two distinct therapeutic payloads on the same antibody, enhancing treatment efficacy by promoting synergistic effects and reducing the risk of drug resistance. However, the synthesis of dual-payload ADCs is complex due to the presence of multiple functional groups on antibodies. In this review, we comprehensively summarize the construction strategies for dual-payload ADCs, ranging from the design of ADC components to orthogonal chemistry. The subsequent sections explore current challenges and propose prospective strategies, highlighting recent advancements in dual-payload ADC research, thereby laying the foundation for the development of next-generation ADCs.

Keywords

Dual payload; Antibody drug conjugates; Bioconjugate; Branched linker; Drug resistance; Combined therapy

1. Introduction

Antibody-drug conjugates (ADCs) represent the rapidly advancing frontier of oncological therapeutics. These agents are characterized by tumor-targeting monoclonal antibodies conjugated to cytotoxic payloads via precisely engineered chemical linkers, enabling both targeted delivery and potent drug efficacy [1]. ADCs offer several advantages over conventional chemotherapeutic agents, including enhanced chemotherapeutic efficacy, lower minimum effective doses, higher maximum tolerated doses, and reduced systemic exposure and toxicity [2]. To date, 13 ADCs have received marketing approval by from the FDA, with hundreds more undergoing clinical evaluation [3]. Economic analyses highlight the rapid growth of the global ADC market, which is projected to reach $11.29 billion in 2023 and grow at a compound annual growth rate of 9.2% between 2024 and 2030 [4]. This emerging drug modality, often referred to as "biological missiles," signals a new era in targeted cancer therapy.

Despite the significant expansion of therapeutic options facilitated by ADCs, several challenges persist. One major issue is the limited penetration of antibodies into tumor tissues, which restricts drug delivery and results in suboptimal efficacy [5]. Additionally, the enrichment of drug-resistant cancer cell populations under therapeutic pressure remains a significant obstacle in conventional ADC therapies. Reliance on a single therapeutic agent creates selective pressure, allowing insensitive tumor cells to survive and proliferate, leading to acquired resistance within tumor tissues [6]. This underscores why many effective small-molecule-based chemotherapeutic regimens involve the co-delivery of two or more complementary drugs with distinct mechanisms of action. These combination therapies have demonstrated some success in reducing drug resistance and improving antitumor efficacy [7,8]. However, research on combination therapies involving ADCs remains limited, with constrained success due to challenges such as overlapping toxicities and pharmacokinetic differences [9]. Moreover, co-administering two single-agent ADCs targeting the same antigen may lead to binding competition, reducing the delivery

efficiency of each payload and ultimately compromising therapeutic efficacy [10]. To address these challenges, employing a single antibody to deliver multiple payloads is expected to enhance tumor accessibility and overcome resistance issues associated with single-agent ADCs [11].

In recent years, dual-payload ADCs have emerged as a promising approach, representing the next generation of ADCs. These ADCs are defined by the simultaneous incorporation of two distinct payloads with different mechanisms of action. By adjusting the combination of cytotoxic agents and modulating the drug-antibody ratio (DAR), this approach holds the potential to significantly reduce drug resistance, mitigate toxic side effects, and enhance antitumor efficacy [11,12]. Moreover, dual-payload ADCs demonstrate more pronounced therapeutic effects and provide superior survival benefits compared to the co-administration of the two individual drugs [10]. Currently, several dual-payload ADCs are undergoing preclinical trials, exhibiting enhanced pharmacological efficacy in cellular and animal models. However, their validation through clinical trials remains critical. Deviations from expected results are common, such as inadequate payload synergy and overlapping toxicity, which can hinder clinical performance. This underscores that designing dual-payload ADCs involves more than simply combining two ADCs; it requires comprehensive coordination and optimization of linkers, payloads, and antibodies. In this review, we first summarize the design strategies for linkers, payloads, and antibodies, then focus on the current chemical toolbox for site-specific conjugation and antibody modification. We also discuss how these strategies combine to generate dual-payload ADCs, with illustrative examples highlighting their efficiency. Finally, we outline the latest advancements and analyze the prevailing challenges in the development of dual-payload ADCs. This review systematically elucidates the technical concepts and construction strategies, aiming to advance the future clinical application of dual-payload ADCs.

2. Construction strategies of dual-payload ADCs

An ADC is composed of an antibody, a linker, and a cytotoxic payload. Ideally,

an ADC should reach its target without releasing off-target payloads and possess sufficient potency to destroy cancer cells without harming healthy cells. Numerous studies indicate that the efficacy of ADCs depends on the complex interplay between the antibody, linker, payload, and the tumor microenvironment. Therefore, each component of an ADC must exhibit specific and precise attributes to ensure optimal effectiveness.

Dual-payload ADCs, as the next generation of ADCs, are designed to use a humanized monoclonal antibody that binds to tumor-specific or tumor-associated antigens while simultaneously carrying two distinct anti-tumor payloads through cleavable or non-cleavable linkers. While the concept of dual-payload ADCs may seem straightforward, it involves much more than simply adding different payloads. Beyond the design principles of conventional ADCs, dual-payload ADCs present more complex requirements that must be carefully considered (**Fig. 1**).

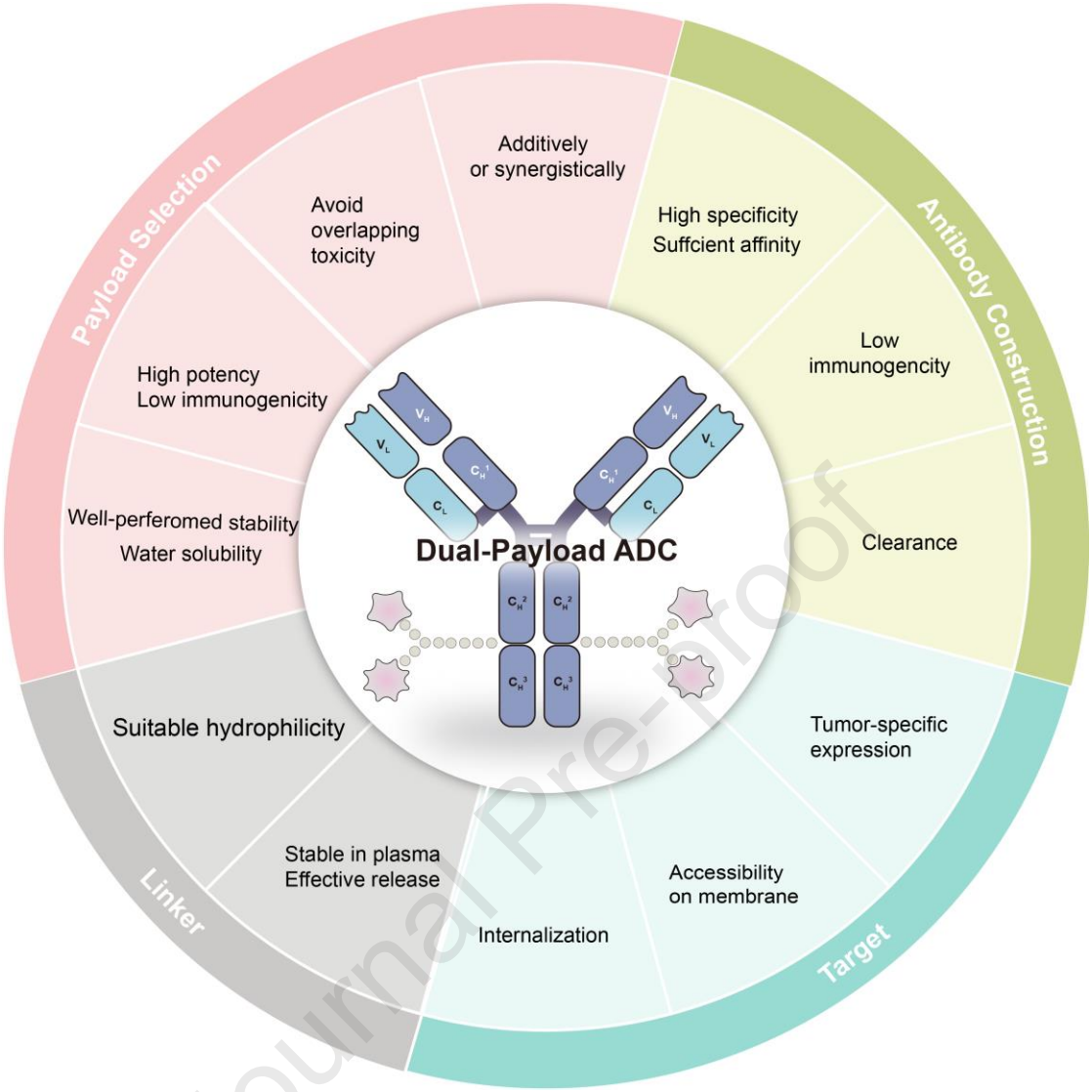

Fig. 1 Desired characteristics of the components of dual-payload ADCs. Each component (the linker, payload, target, and antibody) has a significant impact on the properties of the ADC.

2.1 Design strategies of components of dual-payload ADCs

2.1.1 Selection strategies of targets and antibodies

ADC relies on target antigens and antibodies to recognize cancer cells from normal cells. Therefore, the initial step in the development of a dual-payload ADC is the selection of an appropriate target antigen. As with convention ADCs, to mitigate off-target toxicity and attain an acceptable therapeutic index, the target antigen should ideally exhibit elevated expression levels within the tumor milieu. And it should demonstrate minimal to no expression in normal tissues, or restricted expression to

specific tissue types at the very least. Furthermore, it is imperative that the target antigen is accessible on the cell surface, thereby facilitating the binding of circulating monoclonal antibodies. Preferably, the target antigen should also undergo internalization, as non-internalized ADCs may engender significant toxicity in certain scenarios [13]. And it is better to minimize the shedding of desirable antigens to prevent the sequestration of free antigens by circulating antibodies, thereby affecting the efficacy of the drug [14].

Consistent with the principles of target selection for traditional ADCs, currently validated targets certainly remain viable options for dual-payload ADCs, such as HER2, Trop2, and CD30. Nevertheless, there is more to be considered between the target of the dual-payload ADC and the selected payload combination. It is crucial to ascertain the expressivity of the target antigen and the sensitivity of the cell groups to the chosen drug combinations. This allows for a balance between potency of the drugs and antigenic density of the target, thereby achieving optimal efficacy and safety.

In regard to antibodies, it is essential that they demonstrate an adequate level of affinity and specificity towards the target antigens, in accordance with the fundamental requirements of conventional ADCs. Additionally, their pharmacokinetic attributes should remain uncompromised upon conjugation to payloads [11]. Furthermore, the selected antibodies ought to demonstrate low immunogenicity, appropriate plasma clearance, and swift internalization capabilities, among other essential requisites [15]. The antibodies employed in extant dual-payload ADC studies remain rooted in those utilized for conventional ADCs, predominantly IgG antibodies, encompassing the four isoforms IgG1, IgG2, IgG3, and IgG4. In addition, the integration of novel antibody backbones and target selections is anticipated to broaden the landscape of dual-payload ADCs. Alternative antibody fragments, such as Fab, scFv, VHH, and others, has demonstrated considerable promise [16,17]. Their diminutive molecular sizes can notably enhance tumor penetration and pharmacokinetics, mitigating concerns associated with the larger volumes of mAbs that may impede drug delivery [18]. Furthermore, considering potential safety

concerns associated with dual-payload ADCs, it is imperative to implement a non-specific antibody detection strategy to mitigate the risk of off-targeting.

2.1.2 Selection strategies of linkers

The linker in ADC represents a pivotal connection between the antibody and cytotoxic payloads, orchestrating the release of the payload and ensuring the stability of the drug. Linkers intended for dual-payload ADCs must still adhere to the fundamental principles of balancing plasma stability with precise and efficient cleavage, while also featuring appropriate conjugation sites akin to conventional ADCs [19–21]. As the core of dual-payload ADCs, the conjugation of dual drugs poses significant challenges for linker design. Therefore, innovative linker design strategies are imperative to address these complexities.

Depending on the chosen conjugation strategy, dual-payload ADCs may employ either a traditional linear linker or a branching linker equipped with orthogonal reaction handles (**Fig. 2**) :(i) Dual-site conjugation entails the utilization of two reaction sites for the conjugation of separate payloads during assembly [22]. This approach necessitates a sophisticated amalgamation of various conjugation technologies, which will be expounded upon later in this review. (ii) Single-site conjugation involves the use of a single reaction site, typically an exposed cysteine or disulfide bond, to introduce a multifunctional linker [23]. Single-site conjugation streamlines the selection of the appropriate conjugation region, mitigating the potential for interference phenomena that could impede target binding [24]. This method is applicable to a diverse array of antibodies and obviates the need for intricate recombinant technologies to achieve site-selective conjugation [11]. Moreover, it facilitates an increase in the DAR with minimal chemical or enzymatic modifications to the antibody structure, thereby enabling efficient and precise ADC construction [25].

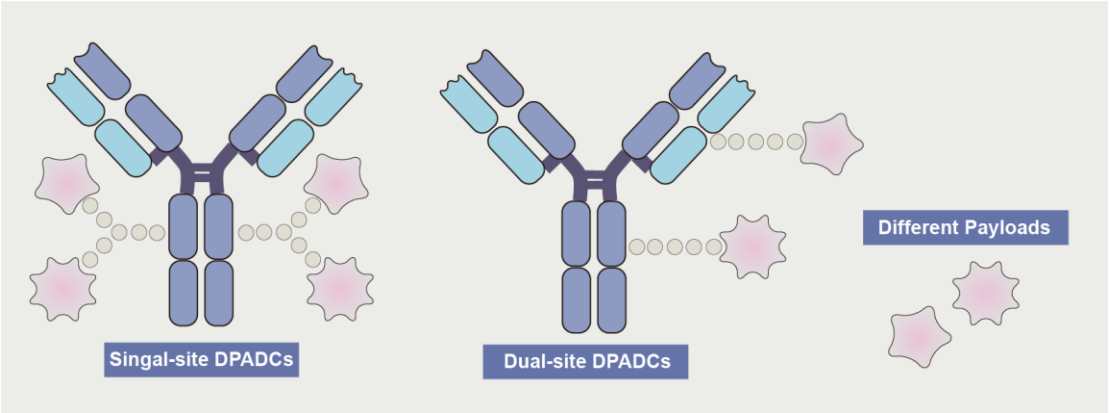

Fig. 2 Two different approaches for dual conjugation of dual-payload ADCs, single-site and dual-site.

Reducing hydrophobicity represents a critical consideration in dual-payload ADCs to optimize their pharmacokinetic profile and therapeutic index. Notably, dual-payload ADCs are readily capable of achieving high DAR. While ADCs with higher DARs are more potent in vitro, antibodies are subject to expedited clearance from plasma, thereby diminishing in vivo exposure and efficacy [26,27]. Research indicates that plasma clearance is positively correlated with the hydrophobicity of ADC. Consequently, the issue can be addressed through the design of hydrophilic linkers [28]. And it has been demonstrated that achieving higher DARs through hydrophilic linker design does not compromise normal pharmacokinetic properties [29]. Furthermore, the reduction of hydrophobicity is of particular significance in single-site conjugation. Single-site conjugation is inherently limited by the proximity of payloads, which are typically highly hydrophobic. This results in a concentrated region of hydrophobicity on the ADC, with the potential for non-covalent stacking interactions between adjacent payloads. This phenomenon leads to increased aggregation, which in turn affects ADC clearance, and ultimately efficacy and safety.[30]

Nevertheless, most payloads necessitate hydrophobic molecules to sustain efficacy, prompting the consideration of hydrophobicity masking as an alternative approach to payload modification [31]. This entails the incorporation of hydrophilic modifiers such as polyethylene glycol (PEG) and polysarcosine (pSar) to conceal the

intrinsic hydrophobicity of payloads [32–34]. These strategies mitigate nonspecific clearance of high-DAR ADCs, yielding dual-payload ADCs with elevated DARs and commendable in vivo performance. However, this does not apply in all cases. The introduction of bulky coiled PEG chains also reduces the efficiency of conjugation, which may limit the localization of linkers to solvent-exposed sites [35].

The choice between cleavable and non-cleavable linkers is a critical consideration in dual-payload ADC design. Cleavable linkers rely on intracellular processes for toxin release, such as cytoplasmic reduction, exposure to acidic lysosomal conditions, or protease cleavage [36]. However, there is a risk of premature breakdown in circulation, which can lead to off-target toxicity and exacerbate safety concerns due to the presence of multiple payloads in dual-payload ADCs [13]. Recent studies, however, have demonstrated the stability of certain cleavable linkers, such as the Val-Cit linker, in circulation, addressing concerns about premature payload release [37]. From a pharmacokinetic standpoint, cleavable linker ADCs are rapidly processed and transported into early endosomes upon uptake. In contrast, non-cleavable linker ADCs require further lysosomal processing for payload release, and alterations in lysosomal delivery in drug-resistant cells may pose challenges for these linkers [38]. Moreover, non-cleavable linkers have been associated with reduced efficacy for certain payloads, such as doxorubicin, monomethyl auristatin E (MMAE), and pyrrolobenzodiazepine (PBD) [39,40]. However, this can be mitigated by optimizing linker design and payload structure [41]. The choice of linker type is intricately linked to target selection, payload choice, antibody properties, and the tumor microenvironment, highlighting the need for a holistic approach in dual-payload ADC design to balance efficacy, toxicity, and drug resistance [42,43].

2.1.3 Selection strategies of payloads

Cytotoxic payloads are crucial in determining both the antitumor efficacy and potential adverse reactions of ADCs. When selecting payloads for conventional ADCs, it is essential to carefully evaluate whether they meet key criteria, including a clear pharmacological mechanism, an effective mode of action, potent tumor cytotoxicity,

high selectivity, robust stability, low immunogenicity, and appropriate hydrophilicity. [44,45].

In addition to meeting the previously mentioned criteria, dual-payload ADCs require the integration of efficient, additive, or synergistic drug combinations to reduce the necessary payload dose for achieving effective cell death [12]. For example, MMAE, known for its cell permeability and bystander activity, is released from the mc-vc-MMAE drug linker. However, MMAE is subject to efflux by multidrug resistance (MDR) exporters and shows reduced activity in cells with elevated pump expression. In contrast, monomethyl auristatin F (MMAF) and cys-mcMMAF, released from mc-vc-MMAF and mc-MMAF ADCs, exhibit reduced susceptibility to drug efflux and maintain efficacy in MDR (+) cells. However, they suffer from limited cellular permeability and lack bystander activity. Thus, the complementary properties of these two drugs may enhance cytotoxic effects in cancer cells when incorporated into dual-payload ADCs [11].

Furthermore, it is imperative to evaluate the potential toxicity of payload combinations. Given that only approximately 2% of ADCs reach targeted tumor sites following intravenous administration [13], it is evident that high potency (IC50 in the nanomolar and picomolar range) is necessary for the compounds to be employed as payloads in ADC [46]. However, two payloads, due to their disparate mechanisms of action, biological activities and pharmacokinetic properties, have disparate effective doses and toxic doses. This can readily result in a situation where one payload has reached its toxic dose while the other has not yet reached its effective dose; or where one toxin has reached its effective dose while the other has exceeded its maximum tolerated dose. Therefore, it may be preferable to utilize appropriate combinations of proven toxic molecules, or combinations of payloads that are less effective as single agents but have a superior safety profile.

Dual-payload ADCs featuring higher drug-to-antibody ratios (DARs) and cleavable linkers may elevate off-target toxicity rates [5]. Thus, in certain instances, a reduced drug load may be preferable, and consideration must be given to potential

overlapping toxicities between the two payload types during the design of dual-payload ADCs. Examples include peripheral neuropathy induced by MMAE and DM1 derivatives, as well as ocular toxicity elicited by MMAF and DM4, among others [47]. Additionally, reducing the cellular permeability of both drugs can help alleviate their associated toxic bystander effects [48].

2.2 Conjugation and modification methods

Drug conjugation can profoundly influence the pharmacokinetics of carrier molecules, potentially impeding in vivo target engagement [49]. Dual-payload ADCs, distinguished by their intricate structure in comparison to conventional ADCs, necessitate stringent criteria for homogeneity and circulation dissociation rate to mitigate off-target toxicity, augment delivery, and enhance efficacy and tolerability.

Conventional stochastic conjugation techniques are simple and widely employed, but yield ADCs characterized by disparate binding sites and DARs, resulting in highly aggregated, heterogeneous formulations fraught with variable pharmacological attributes [50]. Such formulations are prone to impede antibody functionality and induce nonspecific toxicity, thereby affecting drug efficacy and safety [51]. Furthermore, they often exhibit elevated clearance rates, thereby complicating optimization procedure to minimize DAR discrepancies [25]. To surmount these challenges, site-selective conjugation strategies have emerged as pivotal approaches in dual-payload ADC construction, ensuring the attachment of a predetermined number of payload molecules to specific antibody sites. This approach ensures homogeneity and stability of drug during large-scale manufacturing [52]. Homogeneous ADCs synthesized through this methodology broaden robust and consistent pharmacokinetics compared to their heterogeneous counterparts, characterized by heightened affinity, diminished aggregation, and enhanced tolerability [51].

Through site-selective conjugation, a reactive moiety can be selectively introduced at predetermined locations on the antibody surface via chemical modification or genetic engineering techniques, facilitating subsequent coupling to the

toxin molecule. The resultant ADCs exhibit homogeneity and stability, circumventing the pronounced heterogeneity inherent in traditional conjugation approaches and expanding the therapeutic window [53]. We summarize its available chemical toolbox commonly used here, as shown by **Table 1**. For a more detailed examination of site-selective conjugation, we recommend consulting other comprehensive reviews on this subject [54–56].

One such advancement is engineered cysteine approach, which achieves site-selective and homogeneous coupling by introducing cysteine at specific antibody sites. The choice of cysteine introduction site can profoundly influence the pharmacokinetic and toxicological properties of ADCs [57,58]. The conjugation of engineered cysteine residues with appropriate electrophilic reagents has been extensively investigated, with maleimide representing the most commonly employed reagents. However, with the growing recognition that cysteine-maleimide conjugates are prone to decomposition, the study of cysteine conjugation in recent years has yielded several novel linkers that have been used in the synthesis of ADCs. These include self-hydrolysing maleimides [59], iodoacetamides [60], bromomaleimides [61], carbonylacrylic reagents [62] and others, to optimise the synthetic process and product stability. Similarly, selenocysteine-engineered approach operates on a comparable principle. Selenocysteine, structurally analogous to cysteine, possesses exceptional reactivity, enabling swift and efficient reactions under near-physiological conditions. The site-directed incorporation of selenocysteine can be achieved by employing the non-canonical amino acid approach [63].

The non-canonical amino acid (ncAAs) approach integrates diverse ncAAs into antibodies via genetic code expansion (GCE), thereby furnishing chemical handles for subsequent payload conjugation [64]. The incorporation of ncAAs offers great flexibility and precise control, however, there are still some problems with yield, stability, pharmacokinetics and immunogenicity [65]. Efficiently incorporating two or more different ncAAs into an antibody would facilitate the synthesis of more intricate conjugates. This necessitates distinct tRNA/aminoacyl-tRNA synthetase (aaRS) pairs,

each suppressing a distinct nonsense codon without cross-reacting [66]. To date, while several ncAAs have been incorporated [67,68], the utilization of multiple ncAAs to synthesize dual-payload ADCs remains relatively uncommon.

Enzymatic methods are another site-selective conjugation approach that facilitating the conjugation of chemical substrates to specific amino acid sequences within incorporated proteins. Enzymes can either attach the payload directly to a specific site or introduce a reactive function on the antibody, thus further functionalizing the desired payload [69]. A variety of enzymes are suitable for selective coupling, with microbial transglutaminase (mTGase) commonly employed due to its characteristic site-specificity and optimal activity conditions aligning with those of antibodies [70]. And several enzymatic schemes have been devised to selectively modify the N- or C-terminal of the antibody chain, involving the use of formylglycine-generating enzyme (FGE) and sortases. This is typically achieved through genetic engineering of an enzyme-specific recognition tag [71,72].

And disulfide rebridging approach presents a versatile method for site-selective conjugation, achievable without necessitating genetic modifications by leveraging natural interchain disulfide bonds in an IgG1 antibody, allowing for the introduction of biorthogonal functionalities into an antibody [73]. The covalent reconnection of cysteine residues enables the controlled loading of a single drug per disulfide bond, while maintaining the stability of the disulfide bridge. This modification is distal to the antigen-binding or Fc region, thus the antibody's biological activity is less susceptible to impairment [74]. This method mandates the design of appropriate linkers harboring bisulfhydryl reactive groups, such as bissulfone reagents [75], next generation maleimides (NGMs) [76], pyridazinediones (PDs) [77], and divinylpyrimidine (DVP) [78].

Moreover, glycan antibody modification approach exploits the inherent N-glycosylation site N-297 in the CH2 region of IgG heavy chains. Situated distally from the antigen-binding region, this site remains unobtrusive to antigen binding and is conserved across antibody types, rendering the approach universally applicable.

Notably, this method obviates the need for genetic engineering modifications and capitalizes on natural antibody structures [79]. However, the method requires specific reagents, enzymes and enzyme mutants, and the products are limited in drug-carrying capacity. And the typical process requires multi-step glycoengineering with two or more enzymes, which restricts substrate diversification and increases the complexity of the preparation procedure [80].

Table 1 Brief summary of site-selective conjugation approaches commonly used

Conjugation approaches	Examples of methods	Benefits	Limitations	Representative product in clinical trials (ClinicalTrials.gov Identifiers)
Engineered cysteine	① Maleimides ② hydrolysing maleimides ③ Iodoacetamides ④ Bromomaleimides ⑤ Carbonylacrylic reagents	① Homogeneity ② Tuneable reactivity ③ Stability	① Genetic engineering required ② Typically limited to DAR 2	NCT03386513; NCT04086264; NCT03698552; NCT03884517;
Non-canonical amino acid	① P-acetylphenylalanine (pAcF) ② Para-azidomethylphenylalanine (pAMF) ③ Spiro[2.4]hepta4,6-diene-lysine (SCpHK) ④ Cyclopentadiene-lysine (CpHK)	① Homogeneity ② Tuneable reactivity ③ DAR alteration possible	① Genetic engineering required ② Low antibody expression yields ③ Enhanced immunogenicity ④ Enhanced hydrophobicity	NCT04829604; NCT03255070; NCT02864290; NCT03424603;
Enzymatic	① Microbial transglutaminase (mTG) ② Formylglycine generating enzyme (FGE) ③ Sortase	① Homogeneity ② DAR alteration possible ③ Mild reaction conditions	Aglycosylated/deglycosylated antibodies or genetic engineering required	NCT03682796; NCT02122146;
Disulfide rebridging	① Bissulfone reagents ② Nextgeneration maleimides (NGMs) ③ Pyridazinediones (PDs) ④ Divinylpyrimidine (DVP) ⑤ Arylene dipropiolonitrile (ADPN) ⑥ Dibromomethyl heterocycles (C-Lockt™)	① Homogeneity ② Native amino acid sequence and glycosylation ③ No alteration of antibody structure	① Intrachain misbridging ② Typically limited to DAR 4	NCT04084366; NCT04316442;
Glycan modification	① Antibody glycoengineering via oxidation ② Endoglycosidase-mediated glycan remodelling ③ Deglycosylation-remodelling	① Homogeneity ② No alteration of amino acid sequence ③ Stability	① Different methods of purification required for specific reagents and enzymes ② Immunogenic reactions	NCT03700294;

The development of dual-payload ADCs has significantly advanced through the utilization of biorthogonal chemistry, particularly the integration of click reactions. Click reactions represent a suite of modular, stereospecific reactions characterized by straightforward reaction conditions and benign by-products [81]. Their widespread adoption in biological contexts stems from their ability to facilitate biorthogonal, site-specific, and high-yield conjugation under mild conditions [82]. Moreover, click reactions facilitate the modularization and post-functionalization of antibodies without necessitating intricate synthetic procedures or iterative purification steps [83].

The conventional approach to click chemistry, copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction, has historically been employed in constructing certain ADCs owing to its fast reaction kinetics [84]. However, the employment of copper catalysts may entail cytotoxicity and the potential oxidation of specific amino acids on the antibody, thereby precipitating potential immunogenic reactions [85]. To circumvent these problems, strain-promoted azide-alkyne cycloaddition (SPAAC) employs strained alkyne derivatives as reactive partners to obviate the need for toxic catalysts [86]. Nevertheless, SPAAC is constrained by limited water solubility and sluggish reaction kinetics [87]. Conversely, inverse electron demand Diels-Alder (IEDDA) reaction stands out for its swift reaction kinetics, catalyst-free conditions, and impeccable biocompatibility [88].

In recent years, the emergence of "unclickable" bioconjugation methodologies, such as the chemoselective rapid azo-coupling reaction (CRACR), has garnered attention. CRACR, compatible with select click chemistry reactions, facilitates orthogonal dual modification [89]. Other prevalent biorthogonal reactions include oxime and hydrazone ligations, as well as tetrazine ligations [90]. The judicious combination of biorthogonal reactions serves as a robust cornerstone for dual-payload ADC construction, yielding high product yields devoid of cross-reactivity. Several common combinations of biorthogonal reactions are delineated herein (**Fig. 3**).

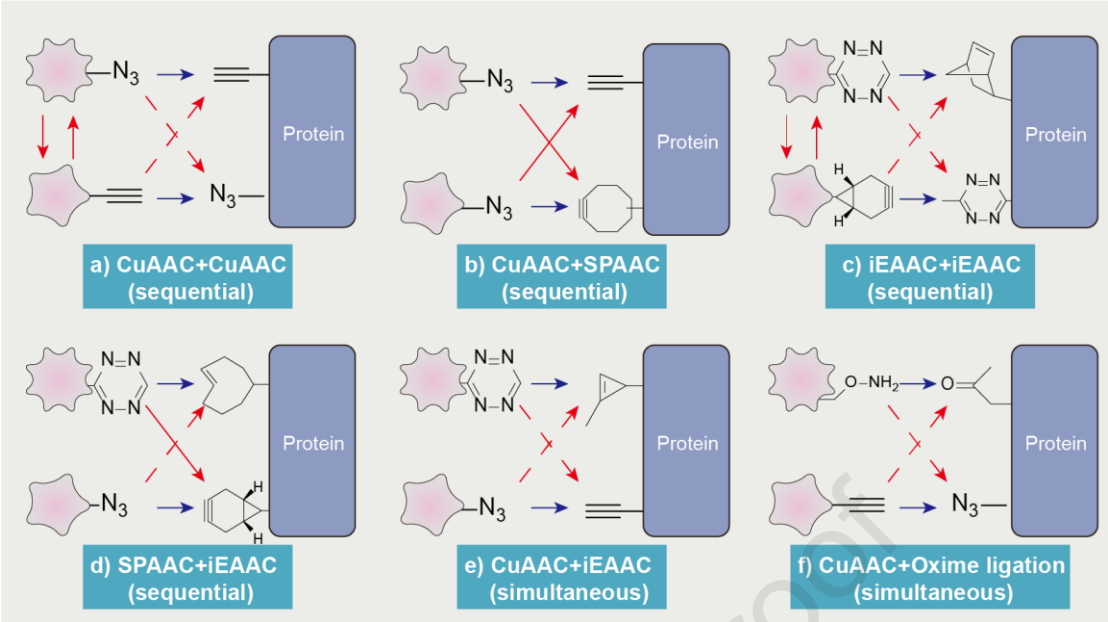

Fig. 3 Bioorthogonal chemistry combinations for dual conjugation of dual-payload ADCs.

3 Current progress of dual-payload ADCs

In recent years, notable strides in dual-payload ADC design have ensued, propelled by the advent of sophisticated antibody modification tools and inventive linker designs, as shown by **Table 2**. This review endeavors to discuss these designs and other relevant studies promising to develop dual-payload ADCs, with a focus on how the above construction strategies were used.

Table 2 List of dual-payload ADC designs

Conjugation strategy	Linker	Linker conjugation	Payloads	Payload conjugation	Antibody	Biological outcomes	Ref.
Single-site	Dual cysteine multiplexing carriers	Classical native cysteine-maleimide conjugation	MMAE (DAR8) +MMAF (DAR8)	Two orthogonally protected cysteine moieties [Cys (SiPr)+Cys (Acm)]	Anti-CD30	Activity exhibited on cell types that are refractory to either of the individual component drugs in mouse xenograph models	[11]
Single-site	Bifunctionalized maleimide heterotrifunctional linkers	Engineered cysteine-maleimide conjugation	MMAE (DAR2) +SG3457 (DAR2)	Alkyne (CuAAC)+ketone (oxime linkage)	Anti-HER2	No additive or synergistic effect.	[84]
Single-site	Bifunctional branched linkers with orthogonal clickable handles	MTGase-mediated conjugation	MMAE+MMAF (DAR2+2/2+4/4+2)	2Azide (with DBCO) + methyltetrazine (with TCO)	Anti-HER2	Greater treatment effect and survival benefit than co-administration of two single-drug variants in xenograft mouse models representing intratumor HER2 heterogeneity and elevated drug resistance	[10]
Single-site	Synthemer platform	(undisclosed)	AF-HPA (DAR2/4/6/12) +I-BiP (DAR2/4)	(undisclosed)	Anti-HER2	No additive or synergistic effect in cancer cell lines	[91]
Dual-site	EDA-Gly3+ 6-aminohexanoic acid linker	Selenocysteine-iodoacetamide conjugation+ engineered cysteine-maleimide conjugation	PNU-159682 (DAR1.9) +MMAF (DAR1.5)	(Drug-linker complexes)	Anti-HER2	No antagonistic or synergistic effect.	[12]
Dual-site	LacNAc-based+ FcBP-TE drug-linkers	Endo-S2-mediated glycan remodeling+ affinity-directed conjugation	MMAE (DAR2) +MMAF (DAR2)	(Drug-linker complexes)	Anti-HER2	No additive or synergistic effect in mouse xenograph models	[92]
Dual-site	(undisclosed)	Enzymatic site-specific conjugation (iLDC/iGDC)	TopoIx+an immune agonist (undisclosed)	(undisclosed)	Anti-Trop2	Leading to an enhanced immunogenic cell death (ICD) and antitumor response in vitro and in vivo via a synergistic MoA.	[93]
Dual-site	(undisclosed)	Two orthogonal enzymatic site-specific conjugations (undisclosed)	TopoIx+an EGFR tyrosine kinase inhibitor (undisclosed)	(undisclosed)	Anti-HER3	Exhibiting robust anti-tumor efficacy in both in vitro and in vivo settings, surpassing single-agent treatments	[94]

This table summarizes construction strategies of dual-payload ADCs with publicly available data.

3.1 Dual-site dual conjugation

Dual conjugation at distinct sites is frequently achieved through a blend of diverse conjugation methodologies. While numerous existing approaches are efficacious, most entail conventional, non-site-specific couplings that could potentially impede binding efficacy [24]. Moreover, many of these techniques rely on cytotoxic metal catalysts such as the CuAAC reaction, which are susceptible to residual metal contaminants, adverse immunogenic responses, and related concerns [95]. To mitigate these challenges, a multifaceted, multi-step process is often imperative for comprehensive characterization, involving separation techniques (e.g., size-exclusion high-performance liquid chromatography), spectroscopic methods (e.g., UV-Vis, mass spectrometry), and size determination techniques (e.g., dynamic light scattering) [96–98].

To optimize the preparation process, strategies such as the site-specific conjugate approach and copper-free orthogonal click reaction can be leveraged. The selection and sequence of two distinct modification methodologies must be meticulously designed to ensure orthogonality, compatibility, and maximal modification efficiency.

Dual conjugation via cysteine and lysine residues. Trastuzumab, a humanized anti-HER2 monoclonal antibody, has been developed to host two distinct payloads through cysteine and lysine residues. Initially, an ADC (Tmab-VcMMAE) was synthesized by conjugating trastuzumab to MMAE via Val-Cit, a cleavable linker. Subsequently, the second payload, DM1, was attached to the ADC via Succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), a non-cleavable linker, yielding the dual-payload ADC (Tmab-VcMMAE-SMCC-DM1). The findings underscore the synergistic and superior cytotoxic effects of the dual-payload ADC compared to a single payload conjugate, devoid of significant toxicity. This method harbors the potential to surmount treatment resistance and thwart tumor recurrence, thereby charting a promising path for employing alternative payloads in dual-payload ADC construction [99].

Dual conjugation via orthogonally reactive arginine and lysine residues.

Previous investigations have showcased the selective modification of two buried lysine residues amidst over a hundred lysine residues in the DVD-IgG1 molecule via hapten-driven conjugation [100]. Building upon this premise, an arginine-lysine site-specific conjugation platform predicated on the modular design of heterodimeric DVD-IgG1 was devised, employing arginine (K99R) instead of reactive lysine. This modification facilitates precise, efficient, and stable drug attachment. The platform facilitates the one-pot assembly of site-specific ADCs hosting two distinct payloads through the orthogonal utilization of h38C2_Lys and h38C2_Arg under mild conditions (**Fig. 4A**). While no experiments were conducted, this methodology holds promise for hydrophilic cargoes soluble in aqueous buffers, such as α-amanitin, PNU159682, and hydrophobic drugs conjugated with polymers. It aligns seamlessly with advanced ADC components, furnishing a convenient platform for dual-payload ADCs and other multifaceted antibody conjugates [101].

In contrast to lysine conjugation, arginine conjugation is hindered by incomplete conversion due to the cellular metabolite methylglyoxal partially blocking the reactive arginine. To enhance conjugation efficiency, a conceptually similar approach was pursued by mutating the reactive lysine of h38C2 to a cysteine. Dual-payload ADCs can be assembled by pairing the mutant h38C2_K99C with either the parental h38C2 or h38C2_K99R, thereby broadening the scope of the h38C2-based site-specific antibody conjugation platform (**Fig. 4B**) [102].

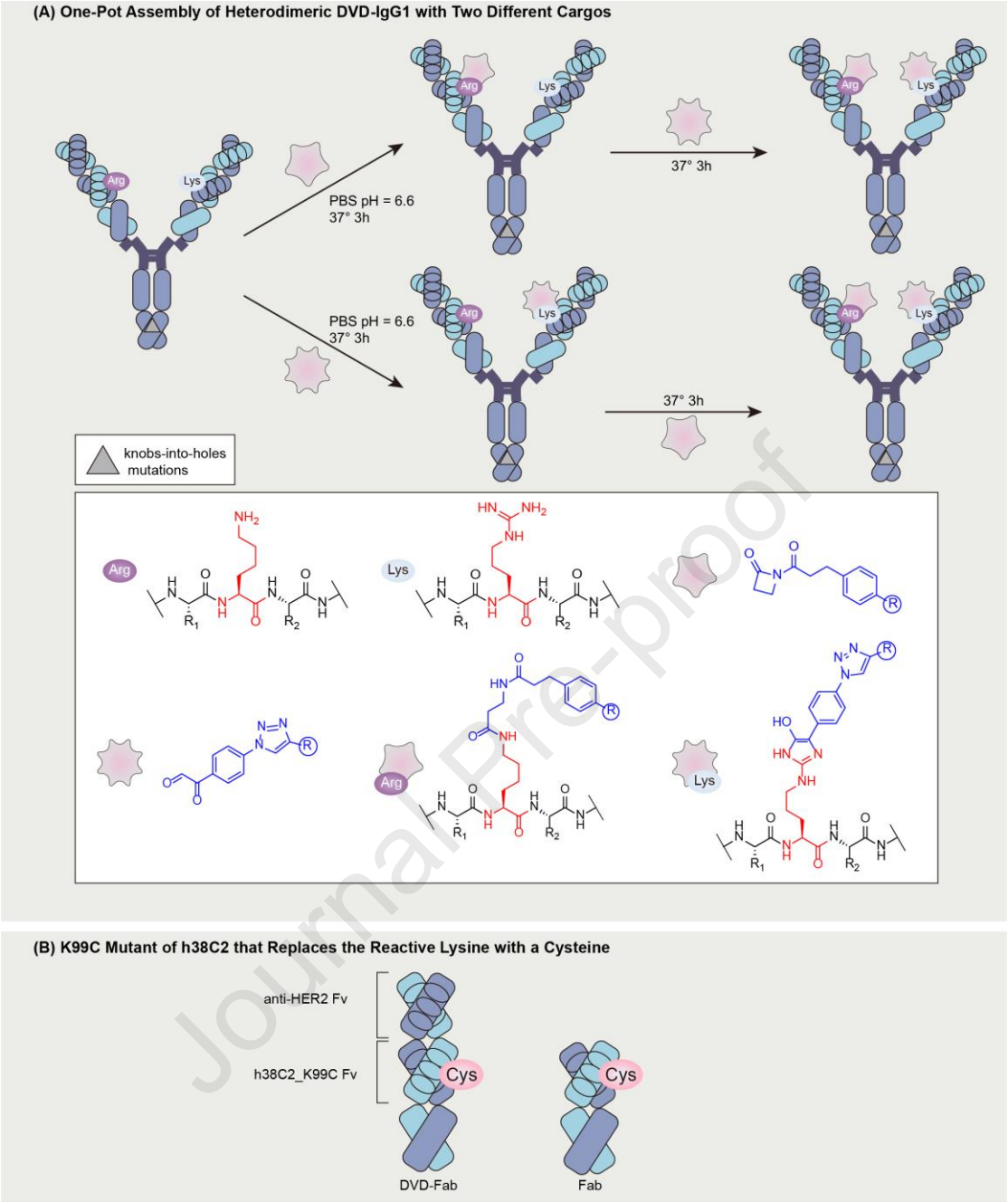

Fig. 4 (A) Scheme of orthogonal labeling of the heterodimeric DVD-IgG1. The conjugation of two fluorescent dyes (pentagram, phenylglyoxal-TAMRA; octagram, b-lactam-hapten-Cy7) was conducted sequentially under the indicated conditions without intermittent purification or buffer exchange steps. **(B)** Mutating h38C2's reactive lysine to a cysteine instead of arginine.

Dual conjugation via the thio-selenomab. The thio-selenomab approach, an amalgamation of engineered cysteine and selenocysteine technologies, was pioneered. Proof-of-concept experiments substantiated that the approach upheld affinity and

internalization, while the modified antibody showcased exceptional stability in plasma [103]. Building upon this innovation, engineered Cys (HC A114C) and Se-Cys (HC S396U) were selectively conjugated at precise sites on trastuzumab. The dual-payload ADC is synthesized by coupling the two entities with iodoacetamide-functionalized PNU-159682 and phenyloxadiazole-MMAF, respectively, thereby furnishing a dual mechanism of action of payloads (**Fig. 5**). However, in vitro cytotoxicity results suggested that MMAF and PNU-159682 do not exhibit synergistic interaction [12].

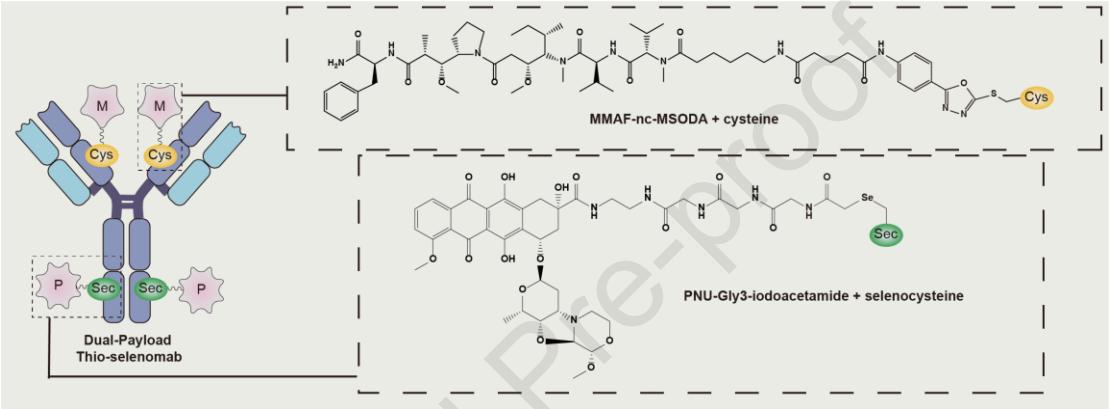

Fig. 5 Dual PNU-159682/MMAF conjugation.

Dual conjugation with cysteine residues and an unnatural amino acid. Utilizing engineered cysteines and unnatural amino acid engineering techniques, Q124C on the light chain and A121 on the heavy chain of CH1 of trastuzumab-Fab (Tra-Fab) were identified as conjugate sites. Subsequently, Tra-Fab underwent conjugation with cysteine and p-acetyl-phenylalanine, respectively, through oxime ligation and Michael addition. This facilitates the sequential concatenation of these two moieties on the Tra-DualFab with two types of payloads (**Fig. 6**). Notably, this method obviates the necessity for a purification step after the initial oxime coupling reaction. The Michael addition for the second ligation can be executed directly without further purification, simply by adjusting the pH of the reaction solution from 4 to 6. Immunofluorescence assays confirmed the complete internalization of Tra-DualFab into target cells. This strategic approach yields newly designed ADCs endowed with functional diversity, the potential to surmount drug resistance, compatibility with multifunctional linkers, and diverse types of payloads [104].

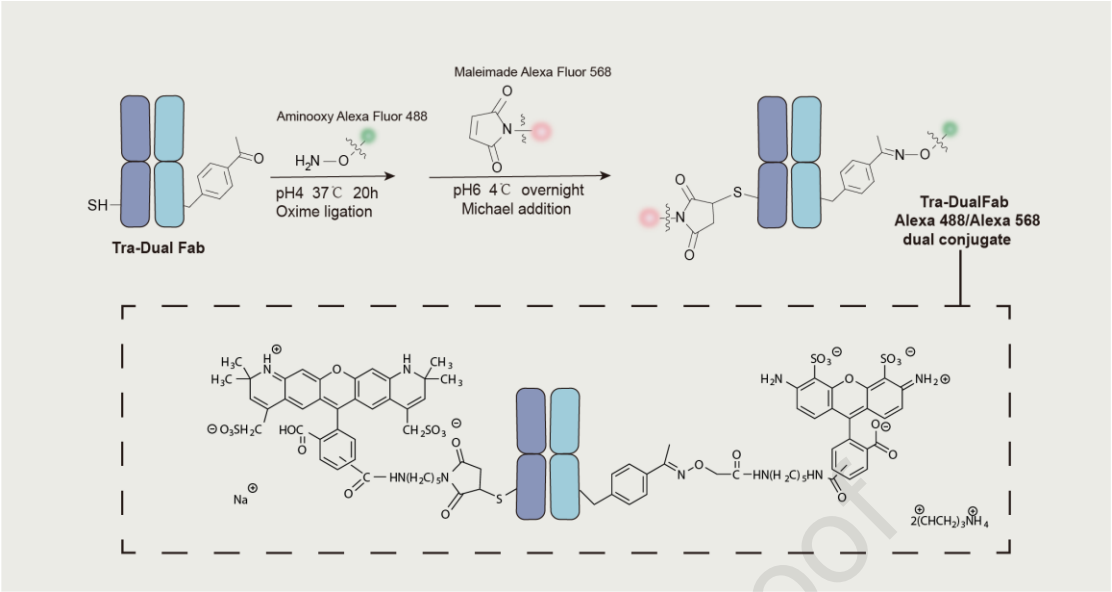

Fig. 6 Schematic diagram of the simple, one-pot, successive method for dual conjugate reactions based on Tra-DualFab.

Dual conjugation via a new dual suppression system leveraging the opal suppressing EcTrp pair. Osgood et al. devised three novel pathways for incorporating dual ncAAs utilizing the bacteria-derived tryptophanyl (EcTrp) pair as a TGA codon repressor, in conjunction with other existing orthologous pairs. They employed the EcTrp + EcLeu pathway to introduce 5-hydroxytryptophan (5-HTP) and the azide-containing amino acid LCA into positions 121 and 198 of the Trastuzumab heavy chain, respectively. Subsequently, the antibodies were conjugated with two distinct cytotoxic drugs, Diazo-MMAF (CRACR) and DBCO-PNU-159682 (SPAAC), employing a one-pot method to fabricate the dual-payload ADC (**Fig. 7**). Cytotoxicity analyses conducted on the NCI-N87 and NCI-H520 cell lines demonstrated that the dual-payload ADC exhibited significantly heightened cytotoxicity against the former, indicative of its HER2-dependent cell-killing potential. This underscores the promise of dual-payload ADCs in therapeutic preparation[68].

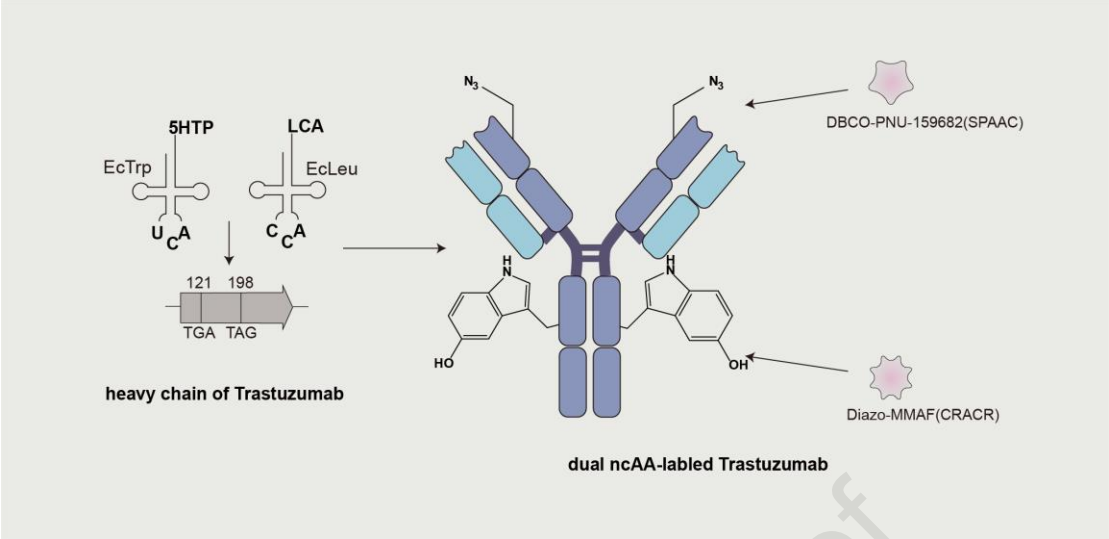

Fig. 7 Site-specific incorporation of two different cytotoxic drugs into dual ncAA-labled Trastuzumab.

Dual conjugation via noncanonical amino acids as doubly bio-orthogonal handles. Previous methodologies for integrating multiple distinct ncAAs at specific sites have proven inefficient and operationally complex. These approaches entail the use of multiple components and involve tedious optimization and evaluation processes, thereby limiting their scalability for therapeutic applications. To circumvent these challenges, two encodable ncAAs, pTAF/mTAF, were devised, each containing mutually orthogonal and bioorthogonal azide and tetrazine "click" handles. Leveraging the technique of engineering unnatural amino acids facilitates one-pot reactions for the introduction of fluorescent groups, radioisotopes, cytotoxic drugs, and other compounds, significantly enhancing conjugation efficiency. The HER2-scFv antibody fragment with pTAF at position A121 was utilized and conjugated with DBCO-PEG3-VC-PAB-MMAE (DBCO-MMAE) via SPAAC reaction, as well as TCO-PEG (with PEG molecules of sizes 20 or 40 K) via IEDDA reaction (**Fig. 8**). In the tumor model, scFv-MMAE-PEG40K exhibited favorable biocompatibility and demonstrated superior anti-tumor activity compared to both the scFv-MMAE and PBS-treated groups. [105].

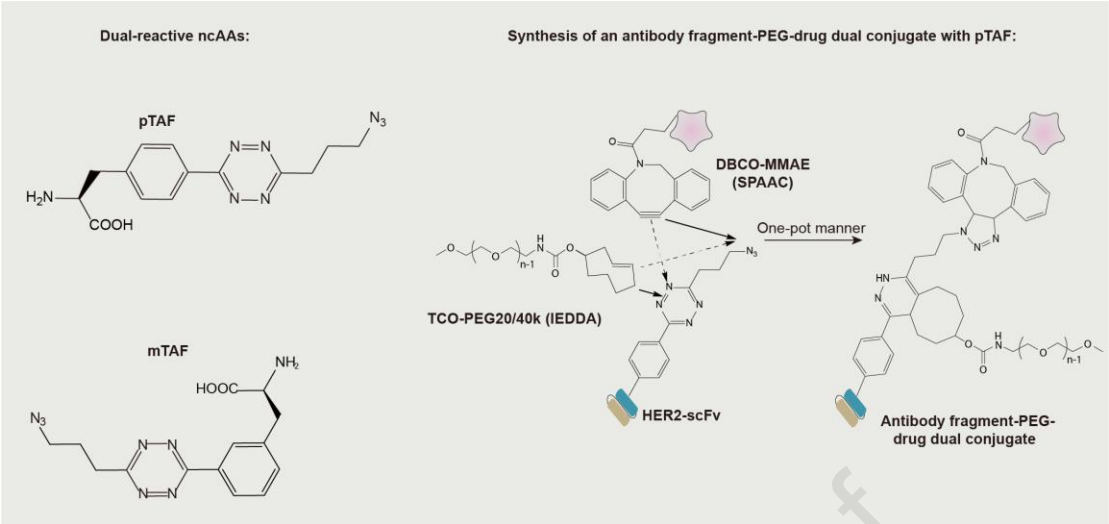

Fig. 8 Chemical structure of pTAF and mTAF; and overview of synthesis of an antibody fragment-PEG-drug dual conjugate with pTAF prepared with DBCO-MMAE and TCO-PEG20K or TCO-PEG40K via SPAAC and IEDDA reactions, respectively.

Dual conjugation via glycan remodeling and affinity-directed traceless conjugation. WeiHuang et al. previously pioneered various strategies for the site-specific construction of ADCs. These include a one-step approach for efficiently producing homogeneous glycosite-specific ADCs utilizing wild-type endoglycosidase endo-S2 [80,106], and a traceless strategy enabling the efficient synthesis of site-specific ADCs through the development of a Fc-directed thioester-based acyl transfer reagent [107]. Furthermore, a straightforward and efficient one-pot technique was devised to assemble site-specific dual-payload ADCs at the conserved N-glycosylation site on Fc Asn297 and K248 site. Two LacNAc-based and two FcBP-based MMAE/MMAF drug linker complexes were formulated, and three synthetic pathways were devised and evaluated. The optimal approach involves initially introducing a single payload, LacNAc-linker-MMAE/MMAF, at the glycosylation site through endo-S2 catalyzed one-step glycoengineering, followed by the installation of another payload at the K248 site using the FcBP-TE-Drug complexes (**Fig. 9**). This methodology enables the synthesis of homogeneous site-specific ADCs with defined DARs or different payloads. These ADCs exhibit satisfactory homogeneity, excellent

buffer stability, and remarkable in vitro and in vivo antitumor efficacy [92].

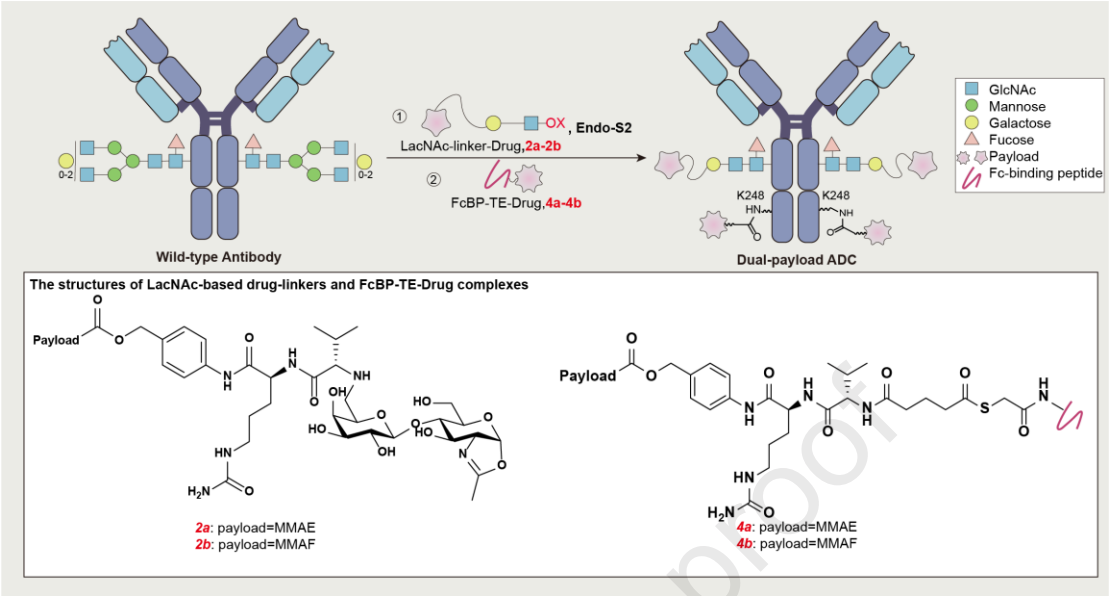

Fig. 9 Schematic overview of synthesis route of dual-payload ADCs with defined DARs; and structures of LacNAc-based drug-linkers and FcBP-TE-Drug complexes.

Dual conjugation via Sortase A-mediated Ligation. Studies have ventured into leveraging human fibroblast growth factor 2 (FGF2) as a target protein for conjugation, diverging from modifying the antibody itself. In 2018, a dual-warhead conjugate combining MMAE and α-amanitin (α-AMTN) was engineered through the Thiol-maleimide and CuAAC reaction (**Fig. 10A**). The resulting dual-warhead-FGF2 conjugate showcased synergistic and potent toxicity of the two loaded drugs, indicating promise in overcoming resistance in the small cell lung cancer line NCI-c. However, the production of such a dual-warhead conjugate posed challenges due to the incorporation of propargyl lysine via CuAAC chemistry and complications in the refolding process of the final product [108].

Building upon this groundwork, MMAE and α-AMTN can be introduced onto FGF2 via an evolved Sortase-A (eSortA)-mediated conjugation reaction (**Fig. 10B**). The resultant dual-payload conjugate exhibited heightened cytotoxic potency compared to the single-loaded counterpart against fibroblast growth factor receptor-positive cell lines. This modular approach ensures site-specific concatenation and

controlled DARs [109]. Recent advancements have extended this strategy to generate dual-warhead cytotoxic conjugates through site-specific conjugation of cytotoxic payloads via SnoopLigase- and evolved Sortase A-mediated ligation. Dual-payload conjugates of MMAE and α-AMTN were prepared using this method, underscoring its potential in constructing dual-payload ADCs analogues (**Fig. 10C**) [110].

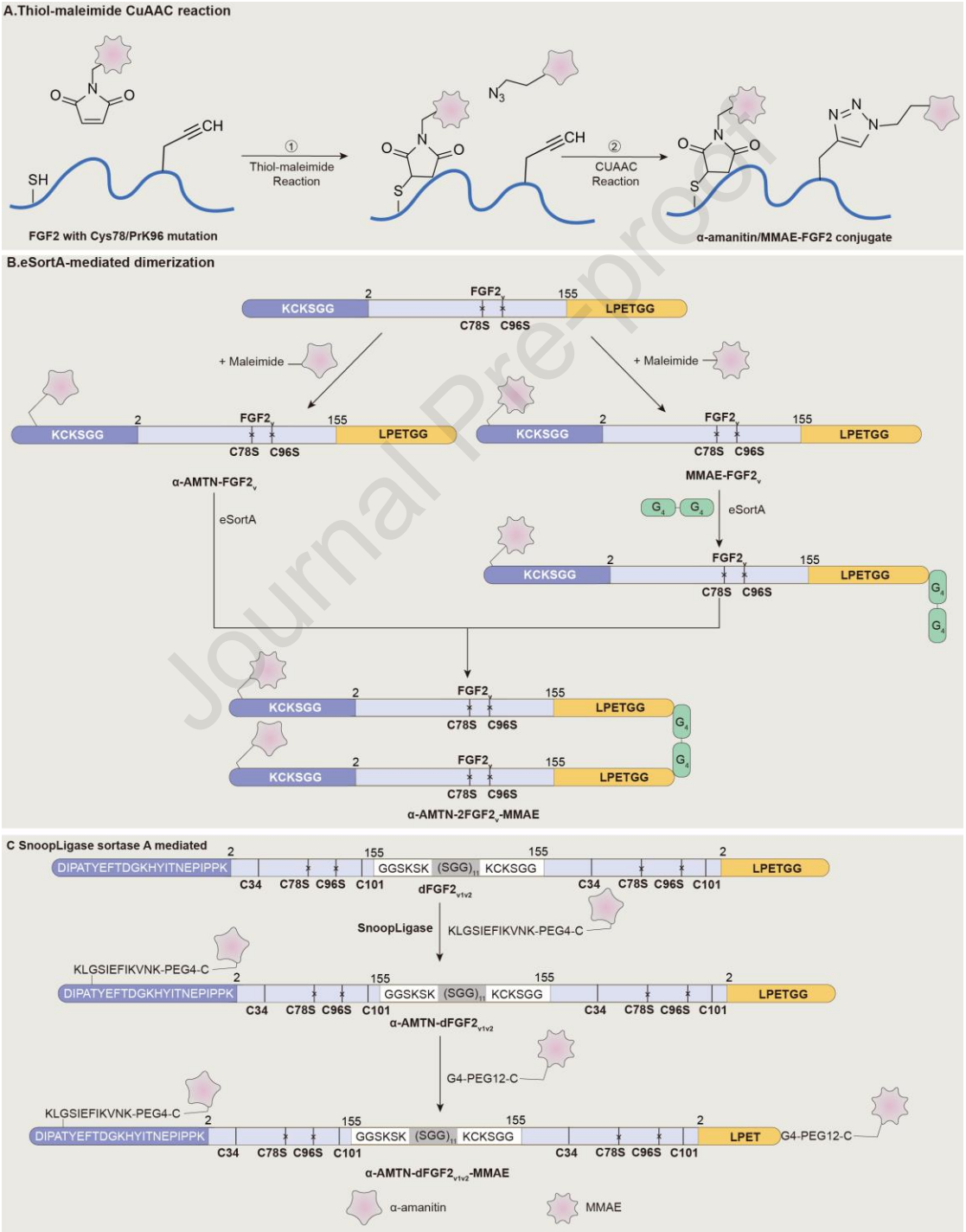

Fig. 10 Dual conjugation via Sortase A-mediated Ligation. (A) Synthesis of FGF2 dual-payload conjugation loaded with MMAE and α-AMTN in a site-specific manner via maleimide-thiol conjugation and Cu(I)-catalyzed alkyne-azide cycloaddition. **(B)** Synthesis of dimeric, dual-payload FGF2 conjugated with MMAE and α-AMTN using eSortA. **(C)** Schematic of the use of SnoopLigase and eSortA in a two-step procedure of the production of dimeric dual-payload FGF2 conjugate.

3.2 Single-site Dual Conjugation

Traditionally, most ADC linkers were tailored to accommodate a single payload, which restricted their versatility and application scope. In contrast, single-site dual conjugation relies on intricately designed linkers capable of regulating DARs with slight modifications to the antibody structure and facilitating more efficient utilization of conjugation sites. But one potential drawback is the issues of hydrophobicity and steric hindrance discussed previously. Moreover, exploration beyond cysteine residue modifications remains relatively underexplored. Single-site dual conjugation can be accomplished through three main approaches: (i) employing reactive linkers pre-functionalized with two cargoes; (ii) utilizing hetero-bifunctional linkers initially functionalized with one cargo and subsequently reacting in a chemoselective manner to introduce the second cargo post-protein conjugation; and (iii) employing hetero-trifunctional linkers. These strategies predominantly target the thiol group of solvent-exposed cysteine residues [24].

Dual conjugation via branched chemical linkers containing two orthogonally masked cysteine residues. Levengood et al. introduced a pioneering approach for preparing single-site dual conjugation ADCs via branched chemical linkers containing two orthogonally masked cysteine residues. In their study, they incorporated two orthogonally protected cysteine moieties [Cys (SiPr)+Cys (Acm)] into natural, non-engineered IgG using a dual cysteine multiplexing carrier tethered with an interchain disulfide bond of the antibody via a maleimide reaction. Subsequent deprotection reactions sequentially unveiled the cysteine residues, which were then conjugated to the payload. This innovative method facilitated the construction of dual-payload

ADCs by uniformly coupling MMAE and MMAF with the CD30-directed antibody cAC10, achieving a DAR of 16 (8 MMAE and 8 MMAF molecules per antibody). Notably, the carrier incorporated a self-stabilizing maleimide (mDPR) for antibody attachment to mitigate drug-linker in vivo deconjugation, along with a PEG24 stretcher enabling high drug loading without inducing hydrophobicity-induced ADC aggregation (**Fig. 11A**). Dual-payload ADCs synthesized using this strategy exhibited activity on cell types that are refractory to either of the individual component drugs in a mouse xenograft model. The versatility of this approach enables efficient screening of antibody and drug-linker libraries to identify dual-payload ADCs with enhanced therapeutic efficacy [11].

Dual conjugation via heterotrifunctional linkers. A bifunctionalized maleimide reagent was developed as a heterotrifunctional linker for site-specific conjugation to cysteine-engineered antibodies through a thiol-maleimide reaction. This reagent features an alkyne group capable of accommodating any azido-decorated drug via a CuAAC reaction, while the ketone group can accommodate an aminooxy-bearing drug via an oxime linkage. The linker was conjugated to trastuzumab (and antibody NIP228 as a control) by introducing cysteine at position 239 of the antibodies through a thiol-maleimide reaction. Subsequently, cytotoxic payloads O-vc-PAB-MMAE and SG3457 were introduced to yield homogeneous dual-payload ADC with a DAR of 4 (**Fig. 11B**). In vitro studies have demonstrated the dual-payload ADC's potent cytotoxicity against HER2+ breast cancer cell lines. While the dual-payload ADC did not exhibit additive or synergistic effects in cell killing, this study illustrates the successful incorporation of two drugs with dissimilar mechanisms of action into a single moiety using a heterotrifunctional linker. This approach opens avenues for creating other dual-modified ADCs utilizing various combinations of drugs with dual mechanistic actions, pharmacokinetic modulating linkers, or secondary targeting molecules with the antibody-linker platform derived from the heterotrifunctional linker [84].

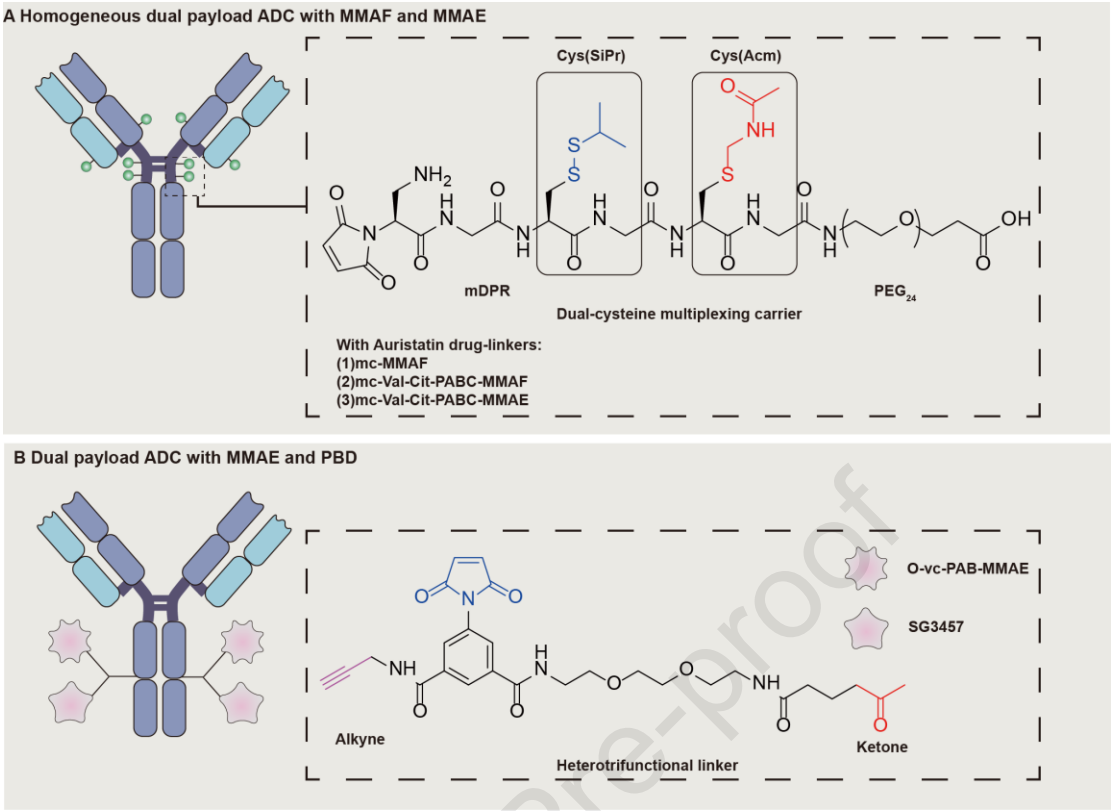

Fig. 11 (A) Homogeneous dual-payload ADCs loaded with MMAF and MMAE and the structure of dual-cysteine multiplexing carriers. **(B)** Dual-payload ADCs loaded with MMAE and PBD, and the structure of a heterotrifunctional linker.

Dual conjugation via DVP disulfide rebridging linkers. Previous studies have highlighted the utility of DVP reagents for the site-specific synthesis of stable ADCs [111]. Building on this foundation, a novel dual functional divinyl pyrimidine (dfDVP) platform has been developed for the synthesis of dual-payload ADCs. The dfDVP reagents are readily synthesized via solid- and solution-phase chemistry. These reagents selectively conjugate one payload by cross-linking two proteinogenic cysteine residues, followed by a bioorthogonal click reaction to attach the second payload. In this study, dfDVP carrying fluorescein isothiocyanate (FITC) and an alkynyl group was synthesized, reacted with the interchain disulfide bond of trastuzumab, and then linked to MMAE via a CuAAC reaction. The resulting Tras-FITC-MMAE, featuring a DAR of 3, displayed significant biological activity in numerous in vitro assays (**Fig. 12**). Neither the antibody nor the payload's activity was negatively impacted. However, despite screening various reaction conditions,

achieving a DAR >3 proved challenging, likely due to the attached molecule's size, permitting only one drug molecule attachment in the hinge region of each antibody [51].

Further enhancement of the dfDVP platform can be realized by introducing IEDDA and SPAAC reactions, yielding an orthogonal bifunctionalized linker containing methylcyclopropene and bicyclononyne handles (**Fig. 12**). This linker can then be reacted with MMAE-PEG4-N3 and TAMRA-PEG4-Bn-Tetrazine after connecting Trastuzumab via a disulfide rebridging technique. Synthesized dual-payload ADC analogs containing enzymatically cleavable MMAE, and fluorescent fractions exhibit a broad therapeutic window against HER2 in SKBR3 (+ve) and MCF7 (-ve) cell lines. This approach offers a modular scaffold's flexibility and the linker's applicability to off-the-shelf IgG1 without requiring protein engineering. Each step proceeds at high conversion rates without the need for metal catalysis and cumbersome purification [78].

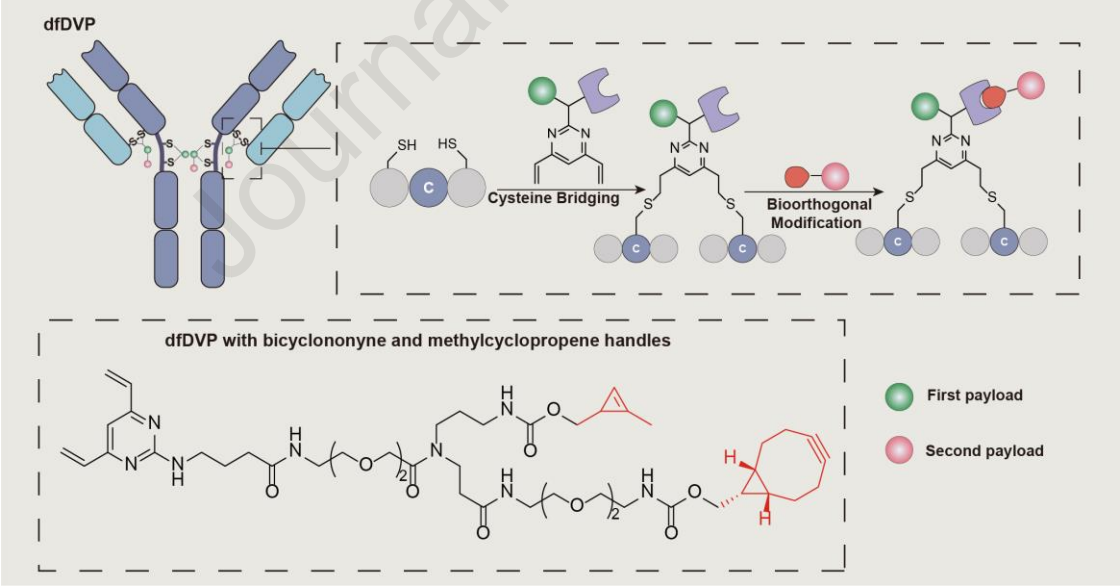

Fig. 12 Schematic overview of dfDVP approach; and the structure of dfDVP with bicyclononyne and methylcyclopropene handles to facilitate sequential SPAAC and IEDDA reactions.

Dual conjugation via pyridazinedione-based trifunctional dual disulfide

rebridging linkers. Pyridazinedione (PD) derivatives present an alternative approach for modifying the interchain disulfide bonds of antibodies, besides the dfDVP approach. In a previous study by Maruani et al., diBrPDs with orthogonal "clickable" handles were incorporated into the natural disulfide bonds of antibody fragments and whole antibodies, aiming to achieve two orthogonal transformations to yield multifunctionalized adducts [112]. Recently, a trifunctional dual-bridging linker, PD-PhN3-Tz, has been developed for the reduction and re-bridging of native proteins with the linker. This linker facilitates the synthesis of a diverse array of ADCs with varying PAR (1, 2, 3 for Fc and Fab′, and 2, 4, and 6 for a mAb) and dual-payload ADCs analogs (2 + 1 for Fc and Fab′; 4 + 2 for mAb) by adjusting the copper-free click reactions employed for model payload attachment (**Fig. 13**). The method can be applied to develop Fab' and Fc-conjugates, as well as coupling full-length antibodies. It offers precise control and versatility regarding accessible PARs and compatible antibody formats, obviating the need for recombinant approach [113].

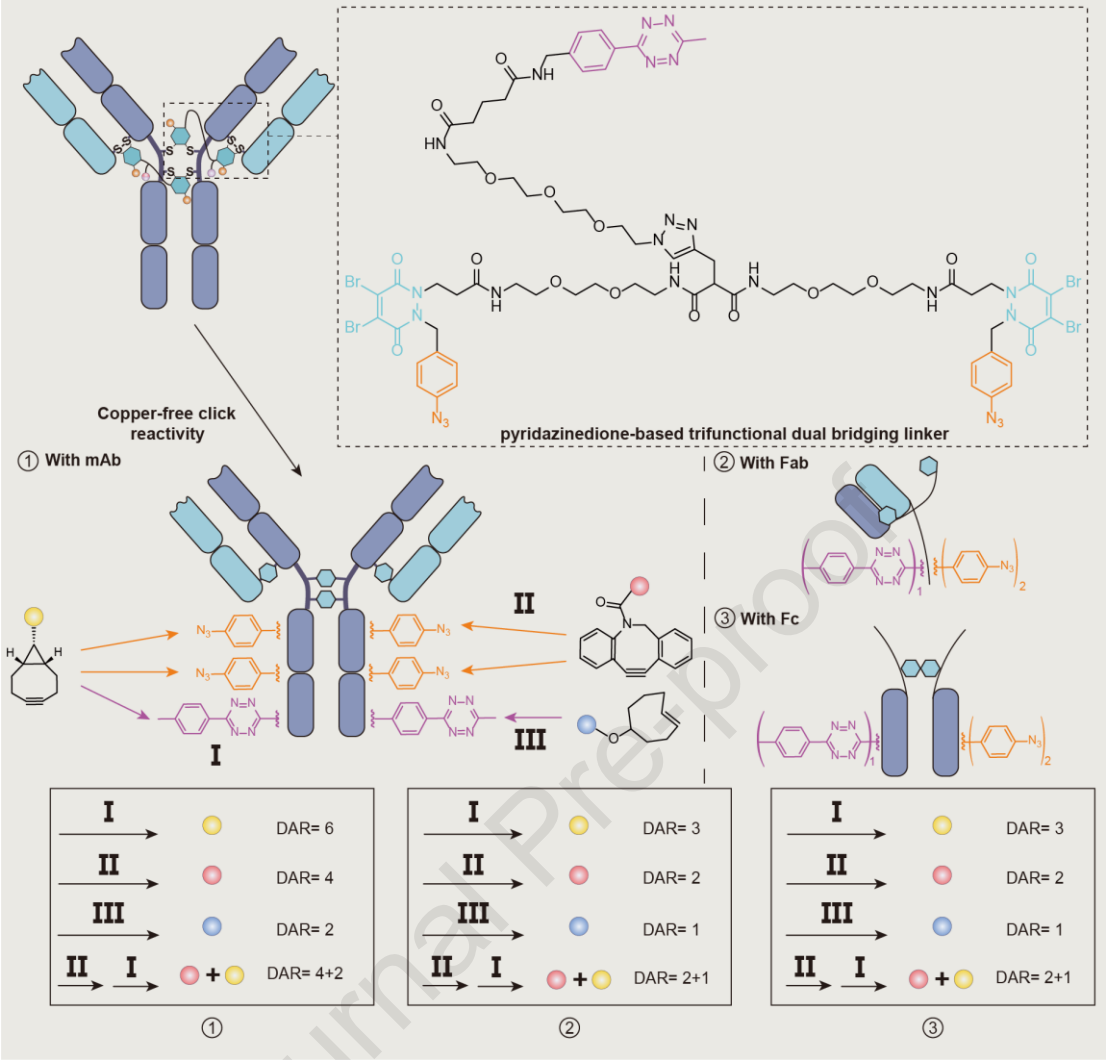

Fig. 13 The structure of a pyridazinedione-based trifunctional dual bridging linker; and its application to construct ADCs with reduction, re-bridging, and functionalisation of mAb, Fab and Fc, enabling introduction of corresponding click handles to controll various PARs.

Dual conjugation via heterobifunctional substrates for MTGase. To achieve one-pot site-specific integration of antibodies, payloads, and bioorthogonal chemical reactive substances, a heterobifunctional substrate for MTGase has been devised. This substrate comprises azide and methyltetrazine "click" handles. Following catalysis by MTGase, the bifunctional linker is introduced into the Q295 site of trastuzumab. This enables the incorporation of DECO-PEG side chains and TCO-DM1 (via SPAAC + IEDDA) through an orthogonal reaction in a one-pot method. (**Fig. 14**) The dual-payload ADC analogs thus synthesized exhibited potent activity in vitro against

SKOV3 cells, underscoring the promise of the dual "click" approach for antibody modification to yield multifunctional ADCs with high drug loading, diagnostic capabilities, and optimized pharmacokinetics [83].

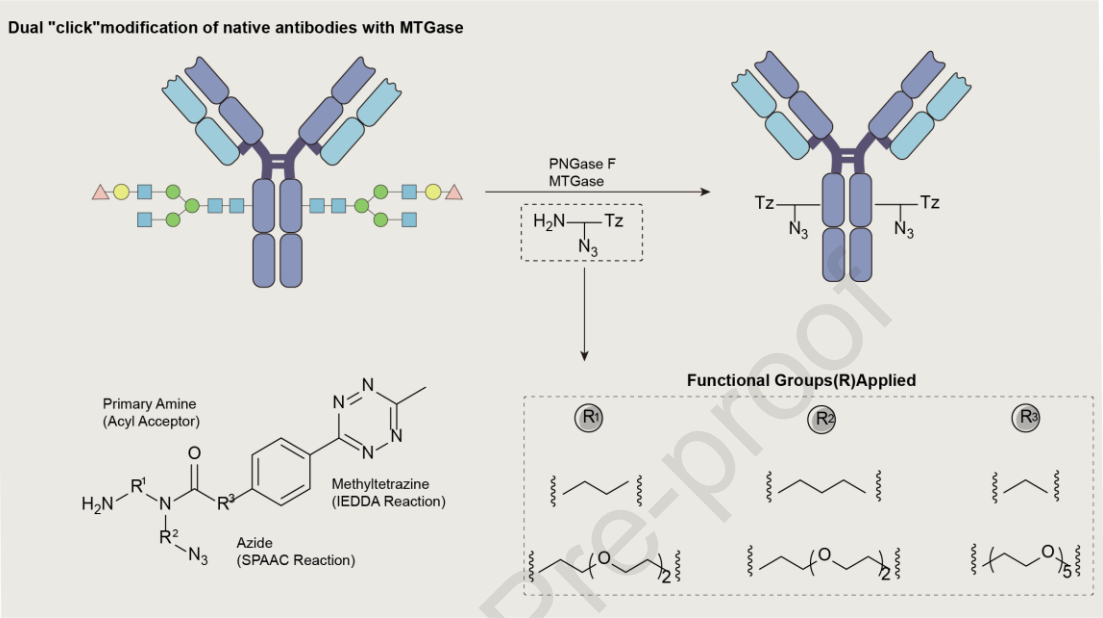

Fig. 14 Schematic overview of site-specific conjugation scheme and structure of heterobifunctional substrates containing two bioorthogonal chemical handles, azide and methyltetrazine.

Dual conjugation via a MTGase-mediated conjugation of bi-functional branched linkers. The Q295 site was utilized to affix a branched linker with orthogonal clickable handles to the N297A anti-HER2 mAb, resulting in a highly homogeneous antibody-linker complex. The anti-HER2 mAb-tri-arm linker conjugate underwent consecutive methyltetrazine-TCO and azide-DBCO cycloadditions in a single pot with TCO-MMAF and DBCO-MMAE modules, producing a series of MMAE+MMAF dual-payload ADCs with DARs of 4+2, 2+2, and 2+4 (**Fig. 15**). These experiments showcased the considerable therapeutic efficacy of these dual-payload ADCs in two mouse models of refractory breast cancer with heterogeneous HER2 expression. Notably, they demonstrated superior in vivo treatment efficacy compared to individual or co-administered single-payload ADCs. The study underscores the therapeutic potential of the homogeneous dual-payload ADC format

in addressing breast tumor HER2 heterogeneity and drug resistance. This process enables precise manipulation of the payload, allowing for control over the type of toxic drug being conjugated as required, as well as regulation of the DAR of each drug to yield ADCs with enhanced homogeneity [10].

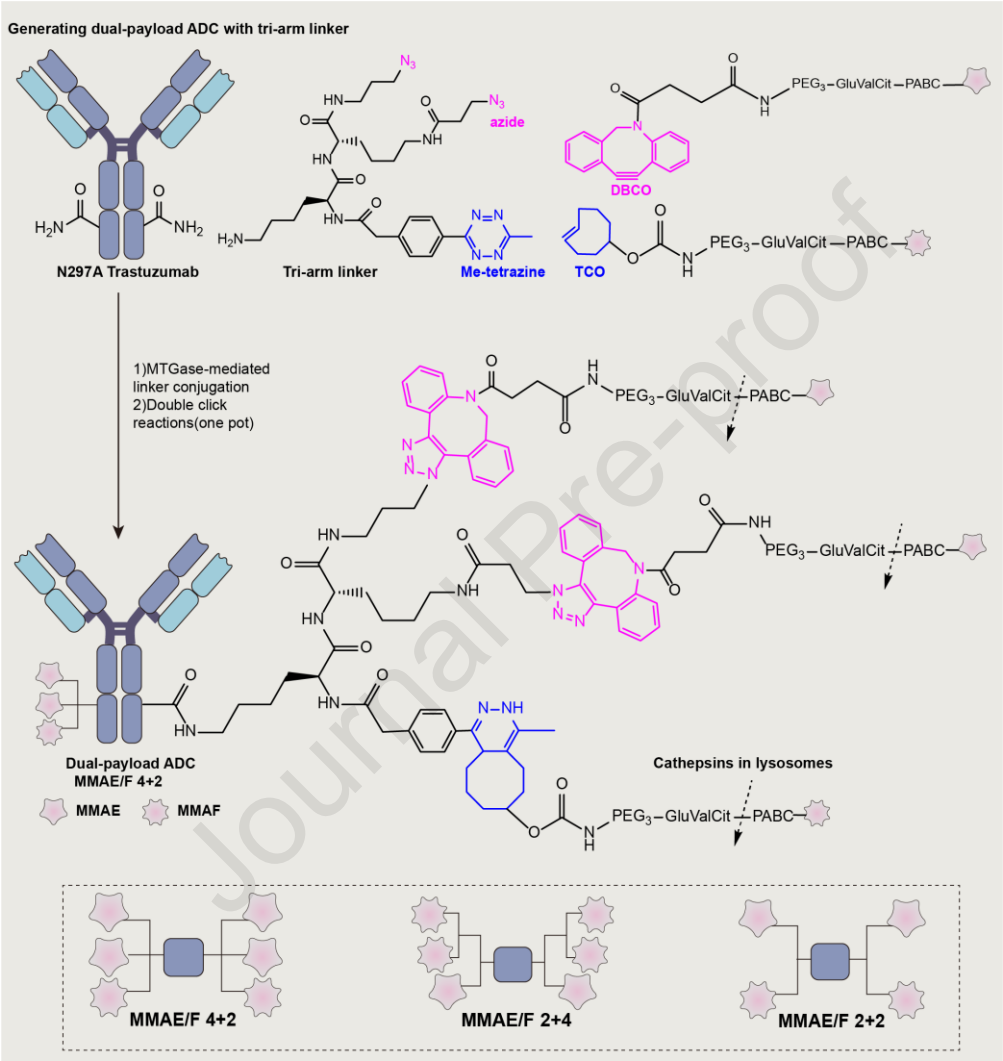

Fig. 15 MTGase-mediated conjugation of tri-arm linkers and following orthogonal click reactions with two payload modules afford homogeneous dual-payload ADCs with defined DARs of 4+2, 2+4 and 2+2. The glutamic acid–valine–citrulline (GluValCit)–PABC linker ensures in vivo stability, while allowing for quick and traceless release of payloads upon internalization and following cathepsin-mediated cleavage in lysosomes.

Dual conjugation via N-terminal cysteines as the minimalistic handle. Dual

conjugation techniques have been broadened to encompass the N-terminal cysteine of a chemically synthesized third-generation anti-HER2 affibody as the reaction locus, extending beyond antibodies. This conjugation methodology leverages the 1,2-aminothiol moiety to concurrently reinstate thiol functionality, facilitating the incorporation of two payloads at the identical site. The process entails a two-step, single-purification approach to introduce DM1-SMCC NHS ester and Val-Cit-PAB-MMAE maleimide, yielding dual conjugates (**Fig. 16**). This dual-payload ADC analogue exhibited heightened cytotoxicity against HER2+ cell lines compared to a combination of two monovalent conjugates, underscoring a potent synergistic effect. Executable under mild conditions, the reaction progresses cleanly, facilitating the extraction of the desired conjugates in substantial yields [24].

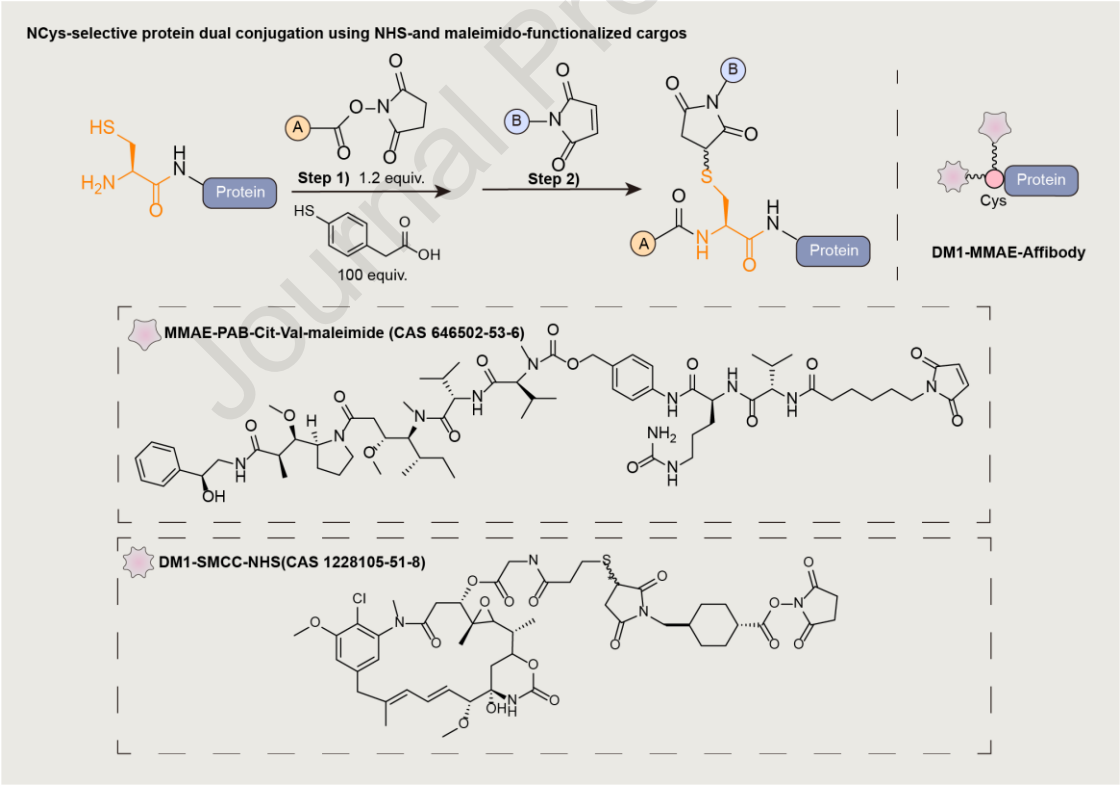

Fig. 16 Schematic overview of NCys-selective protein dual conjugation using NHS- and maleimido-functionalized cargos.

Dual Conjugation via aerobic formylglycine-generating enzymes. A previous study introduced a site-specific concatenation strategy employing FGEs to catalyze

the targeted oxidation of cysteine or serine residues within the conserved (C/S)X(A/P)XR motifs to the aldehyde-containing amino acid formylglycine (FGly) [114,115]. FGEs offer enzymatic diversity applicable for dual site-specific protein conjugations, enabling sequential FGly generation across distinct recognition sequences. This non-traditional electrophilic amino acid can be selectively modified by bioorthogonal Hydrazino-iso-Pictet–Spengler (HIPS) or Knoevenagel ligations to attach payloads, such as fluorophores or drugs, to the protein, achieving a defined payload-to-protein ratio [116].

Despite the benefits of dual bioconjugations with enzymatic orthogonality, the anaerobic enzyme AtsB presents a challenge. Hence, a novel pathway has been proposed to achieve biofunctionalized drug couplings utilizing different types of FGEs for direct dual bioconjugation with orthogonal attachment. DARPin and scFv425-Fc were equipped with two aldehyde tags (CTAGR/CTPSR) and subjected to HIPS ligation and copper-free click reaction. This sequential process can link two distinct payloads to fabricate the dual-payload ADC analogue of MMAE and carboxyfluorescein (**Fig. 17**). Leveraging two O2-dependent FGEs, this method converts two aldehyde tags in a targeted and stepwise manner under mild conditions suitable for natural biomolecules. It facilitates the specific concatenation of peptides and proteins, enabling the construction of dual-payload ADCs and their analogues with a high degree of orthogonality in substrate recognition [117].

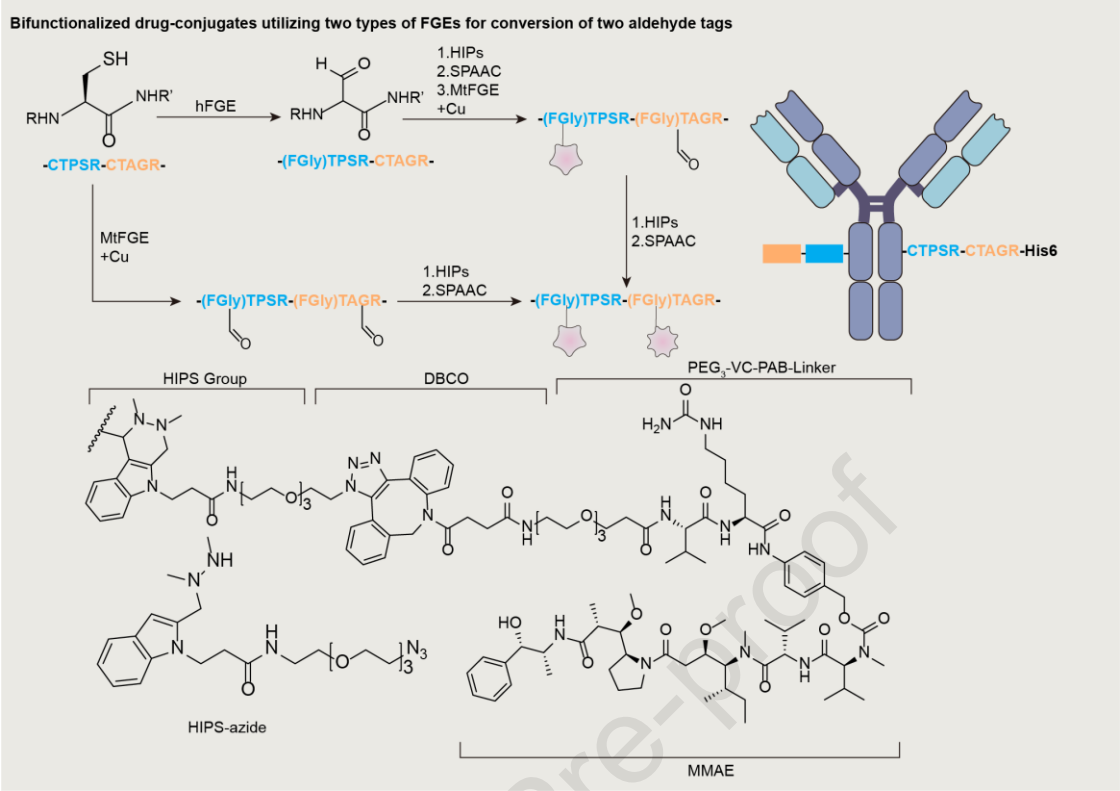

Fig. 17 Combination of Hydrazino-iso-Pictet-Spengler ligation and copper-free click reaction for dual bioconjugation on FGly; and structures of the construct DBCO-PEG-VC-PAB-MMAE connected to the HIPS moiety by SPAAC between the azide and DBCO.

Dual conjugation via bifunctional clickable HIPS and tandem Knoevenagel building blocks. Techniques for conjugation that selectively process FGly via HIPS or Knoevenagel ligation need to address several drawbacks. These include the requirement for a large excess of conjugation building blocks, relatively low reaction rates, and the limited stability of FGly-containing proteins [118,119]. To address these issues, a bifunctional linker for formylglycine affixation has been developed. This linker comprises bioorthogonal reactive HIPS and Knoevenagel affixation modules. It can be used in conjunction with SPAAC for efficient and selective single or dual derivatization with stoichiometric DBCO-modified payloads. This method is ideal for generating polyethylene glycolated therapeutic proteins with improved pharmacokinetics or for creating homogeneous ADCs with DARs of 2 or 4 [119].

Karsten et al. utilized a modular affixation system comprising of FGE and

tandem Knoevenagel ligation in combination with SPAAC to introduce aldehyde groups on DARPin monomer, DARPin dimer, DARPinFc, scFv425Fc. These were then reacted to yield highly homogeneous dual-payload ADC analogues containing PEGylation and MMAE, based on the studies mentioned above (**Fig. 18**). The conjugates inhibited the cell viability of epidermal growth factor receptor (EGFR) overexpressing A431 cells with sub-nanomolar cytotoxicity. They also demonstrated receptor-mediated endocytosis upon receptor-specific binding, indicating the potential of this conjugation strategy to be potent and applicable to a wide range of targeting molecules [120].

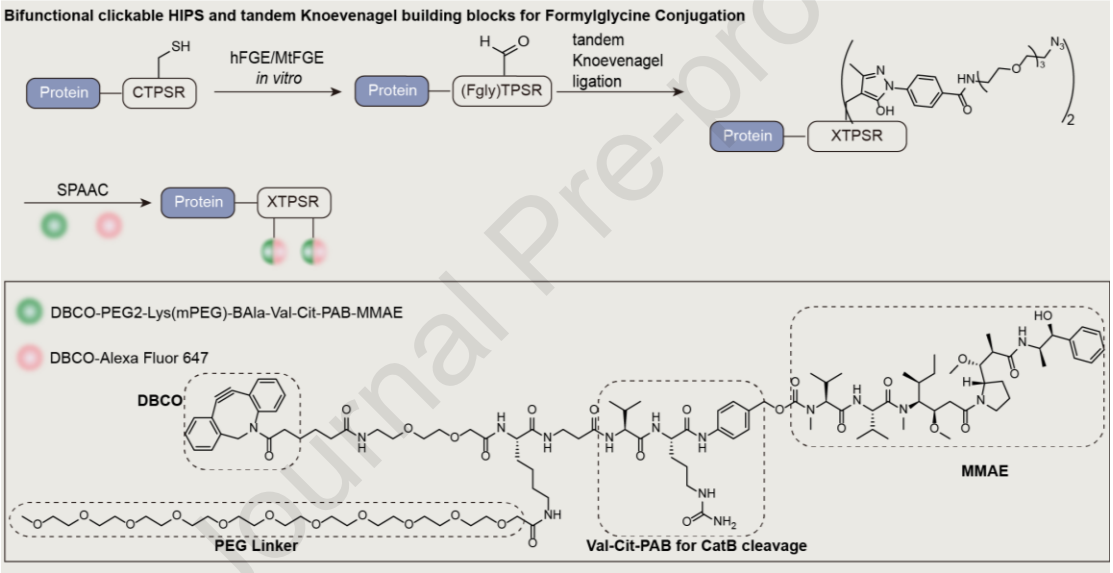

Fig. 18 Schematic figure of enzymatic conversion with the FGE followed by tandem Knoevenagel ligation and strainpromoted azide-alkyne cycloaddition (SPAAC), preparing protein-drug/dye conjugates; and structures of PEGylated DBCO-MMAE linker.

4 CONCLUSIONS AND PERSPECTIVES

ADCs represent a rapidly advancing and remarkably efficacious cohort of anticancer therapeutics, adept at administering potent cytotoxic agents precisely to tumor sites while sparing healthy tissues, thereby embodying a promising amalgamation of chemotherapy and immunotherapy [121]. Nevertheless, as is the case with the majority of cytotoxic drugs, the clinical benefit derived from ADCs as

monotherapies is constrained by the emergence of resistance mechanisms [122]. While the precise mechanisms underpinning drug resistance remain the subject of ongoing investigation, it is understood that tumor cells resistant to ADCs retain susceptibility to alternative ADC formulations and conventional chemotherapeutic agents [123]. In an endeavor to attenuate the likelihood and decelerate the emergence of drug resistance, combination therapy for ADCs is an area of widespread interest. To date, ADCs have been combined with a number of different cancer treatments, including chemotherapy, endocrine therapy, radiotherapy, molecular targeted cancer therapy and immunotherapy [47]. Although this approach demonstrates considerable promise in vitro, its clinical application frequently encounters challenges. While efficacy is commendable, treatment is often discontinued due to serious adverse effects that diminish patients' quality of life [124]. An inherent limitation contributing to this is the excessive discrepancy in pharmacokinetics between ADCs and small molecule drugs [9]. This discrepancy effectively precludes the possibility of simultaneously targeting the same tumor cells, thereby impeding the ability to regulate toxicity of drug combination and resistance of tumors. The combination of two single-payload ADCs is confronted with additional challenges. The binding of two ADCs to the same antigen may result in competition for binding sites, which could potentially affect the delivery efficiency and therapeutic effect [10]. Therefore, dual-payload ADCs represent an effective alternative strategy for ensuring the precise and simultaneous delivery of payload combinations to the same cell, which effectively circumvents the aforementioned problems, offering a promising prospect.

Currently, most advancements in dual-payload ADC development remain at the preclinical stage. As previously mentioned, several preclinical studies on dual-payload ADCs have been conducted by academic laboratories. Within the pharmaceutical industry, Sutro Biopharma unveiled pioneering data in 2013 on a dual-payload ADC, utilizing a monoclonal antibody augmented with two non-natural amino acid residues, followed by the conjugation of distinct payloads through a click chemistry reaction [125]. In 2016, Synaffix secured a patent for its HydraSpace technique, enabling the

site-specific conjugation of a monoclonal antibody with two hydrophilic linkers carrying different payloads [126]. In 2019, Mersana Therapeutics designed a dual-payload ADC containing MMAF and I-BiP, using the Synthemer platform with a DAR of up to 12. Recently, GeneQuantum employed two orthogonal enzymatic site-specific conjugation methods and stable linker technologies to generate a HER3 dual-payload ADC containing TopoIx and an EGFR tyrosine kinase inhibitor [94]. Additionally, some companies have disclosed further information on dual-payload ADCs through patents, such as Aarvik Therapeutics (US20230212181A1) and Novartis (WO2023225359A1).

While various design strategies have been proposed, the potential of dual-payload ADCs remains in the early stages. Numerous challenges must be addressed before clinical application, including issues related to developability, safety, and designs. Developability is a key concern, as few molecules possess biophysical properties suitable for clinical or even preclinical studies. Early screening and evaluation of components can conserve resources and prevent costly late-stage failures. Current dual-payload ADC development tends to be conservative, often relying on validated targets and antibodies. However, matching biomarkers to the mechanism of action of the warhead remains crucial for maximizing the therapeutic index of ADCs [127]. Developing flexible synthesis platforms, such as the click chemistry assembly platform, will facilitate the creation of larger ADC libraries and accelerate the screening process. In addition, particular attention must also be given to the selection of payload combinations. Several reports indicate that although some dual-payload ADCs incorporate payloads designed to exert complementary mechanisms of action, neither payload has demonstrated superior efficacy compared to the corresponding single-payload ADC [12,91]. Given the issue of unanticipated toxicity, drug combinations that yield synergistic outcomes represent the optimal choice. However, it is important to note that certain drug combinations lacking observable additive or synergistic effects may still offer benefits across broader patient cohorts due to interindividual variability [128]. It is therefore crucial to ensure

a balance of efficacy between the two selected payloads and to optimize the DAR to achieve the best therapeutic outcome. A comprehensive toxicity assessment is imperative, including the evaluation of non-linear superimposed toxic effects. Toxicological modeling of dual-payload ADCs must accurately predict combined toxicity and potential long-term effects to determine the maximum tolerated dose and therapeutic window [129].

The lack of in vivo biodata intensifies safety concerns for dual-payload ADCs. While these ADCs hold promise for enhancing efficacy and mitigating drug resistance, their multifaceted pharmacological effects and elevated DARs may lead to adverse reactions and synergistic toxicities. Additionally, the complex modification of antibodies in dual-payload ADCs introduces the risk of altering antibody properties, potentially affecting stability, immunogenicity, off-target effects, and other unforeseen issues [129]. It would benefit from a comprehensive in vivo assessment of efficacy, which necessitates systematic quantitative modeling in toxicology, pharmacology, and pharmacogenetics. Accordingly, antibodies, linkers, and payloads should be optimized synergistically to achieve a balance between efficacy and safety. Furthermore, pharmacogenomic analysis in early clinical trials presents a promising approach to optimizing drug dosing and selection for individual patients, thereby maximizing efficacy and minimizing toxicity [130].

There are inherent flaws in various dual-payload ADC design strategies. As previously mentioned, single-site conjugation emphasizes the development of innovative linkers, commonly based on maleimide, disulfide rebridging linkers, mTG, and other modalities. The primary disadvantages are related to hydrophobicity and steric hindrance, which arise from the close proximity of the payloads [30]. Additionally, there are challenges in manufacturing control due to the multiple purification steps involved [104]. Therefore, innovative linker design, the development of hydrophilic payloads, and the use of hydrophobic masks are challenges that need to be urgently addressed. As for dual-site conjugation, it lies in the combination of different conjugation modalities, requiring the deployment of

multiple orthogonal site-selective modification strategies [22]. The majority of methods currently employed in this field rely on engineered cysteines, non-canonical amino acids, and enzymatic techniques, with further combinations yet to be explored. Given that dual-site conjugation involves the sequential employment of multiple modification strategies, it presents additional manufacturing challenges [131]. Requiring multiple structural changes to the antibody, the risk of altered in vivo properties of the product is particularly high. The disadvantages of certain conjugation techniques, such as the risk of immunogenicity associated with the introduction of unnatural traits through genetic engineering, may become more pronounced [132]. We anticipate that with increased research and data sharing, more systematic and quantitative research models will become available to assist in identifying the most effective design strategy.

With the evolution of medicinal chemistry research methods and the continued in-depth exploration of ADCs, emerging technologies are becoming effective enablers for the development of dual-payload ADCs. With more sequence information and biophysical data becoming publicly available, the task of establishing guiding principles on developability of dual-payload ADCs with artificial intelligence technology is becoming more approachable [133]. The development of AI technology for neoantigen identification and antibody design is surging forward, showing great potential for innovative antibody linker design of dual-payload ADC [134]. For instance, the Response Algorithm for Drug Positioning and Rescue (RADR®) from Lantern Pharma is an AI platform capable of integrating biological datasets to identify ADC targets with improved tumor selectivity, facilitating the rapid development of novel ADCs [135]. Additionally, in drug combination, it has been previously determined through clinical trials. However, this approach has proved to be expensive and time-consuming. High-throughput screening (HTS) is another approach, with challenging to measure the entirety of the combinatorial space [136]. Currently, AI technology has potential to expedite the process of drug formulation design and to facilitate the investigation of drug synergies. Jeon et al. proposed an in-silico method

for personalized drug combination therapy discovery, utilizing genetic information, drug targets, and molecular data from malignant cell lines to estimate the synergistic effects between two payloads. The proposed model was proved to be applied to patient samples in place of cells when it is employed in a clinical setting [137]. And Kaur et al. proposed a unique deep bidirectional mixed density network (BMDN) model capable of visualizing pharmacological synergisms. A dynamic mutation-based multi-objective differential evolution approach is employed to optimize the hyper-parameters of BMDN, with a high degree of accuracy in predicting drug synergy [138]. And multi-tasking deep neural networks can also efficiently predict toxicity in vitro, in vivo and clinical platform, improving the accuracy and interpretability of toxicity predictions [139]. Furthermore, in ADC drug discovery and efficacy validation, traditional cell line models cannot fully replicate the tumor immune microenvironment and tumor heterogeneity. The emergence of functional assays, represented by anthropomorphic models such as patient-derived organoids (PDOs) [140], patient-derived xenografts (PDXs) [141], and conditional reprogramming (CR) cells [142], has provided new opportunities for dual-payload ADC research, addressing the lack of in vivo data. These models facilitate the study of the complex in vivo mechanisms of dual-payload ADCs, maintaining a high degree of consistency with the structure of human source tissues, molecular characteristics, and drug responses.

Dual-payload ADCs epitomize a novel generation of ADCs poised to confer multifaceted therapeutic efficacy, augment ADC potency, and enhance the therapeutic index by harnessing the additive or synergistic effects of diverse payloads they carry. This methodology has exhibited remarkable potential in surmounting tumor heterogeneity and circumventing drug resistance. Despite encountering substantial challenges, as a promising conceptual frontier, it is growing rapidly to surmount the therapeutic challenge encountered by conventional ADCs. Moving forward, it should persist in selective and quantitative coupling methodologies while evolving to embrace universality across a broader spectrum of targets and antibodies. In tandem

with dual-payload ADCs, a range of novel ADC design strategies has emerged, encompassing bispecific ADCs [143], conditionally active ADCs [144], immune-stimulating ADCs [145], protein-degrader ADCs [146], and others. Each variant possesses distinctive capabilities to confront known and potential challenges. The integration of their design paradigms and therapeutic modalities has the potential to facilitate the development of more effective, safe, and personalized cancer treatments.

CRediT authorship contribution statement

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

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

ADCs,
: Antibody-drug conjugates

mAbs,
: Monoclonal antibodies

FDA,
: U.S. Food and Drug Administration

DAR,
: Drug-antibody ratio

PEG,
: Polyethylene glycol

pSar,
: Polysarcosine

MDR,
: Multidrug resistance

MMAE,
: Monomethyl auristatin E

PBD,
: Pyrrolobenzodiazepine

MMAF,

Monomethylauristatin F

IC50,
Half-maximal inhibitory concentration

VHH,
Variable domain of heavy chain of heavy chain antibody

scFv,
Single chain antibody fragment

ncAAs,
Noncanonical amino acids

NGMs,
Next generation maleimides

PDs,
Pyridazinediones

DVP,
Divinylpyrimidine

CuAAC,
Copper(I)-catalyzed azide-alkyne cycloaddition

SPAAC,
Strain-promoted azide-alkyne cycloaddition

IEDDA,
Inverse electron demand Diels-Alder

CRACR,
Chemoselective rapid azo-coupling reaction

aaRS,
Aminoacyl-tRNA synthetase

HIPS,
Hydrazino-iso-Pictet–Spengler

AF-HPA,
Auristatin F hydroxypropyl amide

Data availability

No data was used for the research described in the article.

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Highlights

1. Summarize construction strategies of dual-payload ADCs comprehensively.

2. Introduce currently available chemical toolbox for dual-payload ADC design.

3. Summarize the latest progress in dual-payload ADCs.

4. Propose appliable suggestions for further development.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: