

The Site-specific Conjugation Strategy of HER2-targeted Antibody-drug Conjugates and its Application in Tumor Treatment

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Abstract: This paper focuses on the site-specific conjugation strategies of HER2-targeted antibody-drug conjugates (ADCs) and their applications in tumor therapy. First, it elaborates on the biological characteristics of HER2-positive tumors, the current treatment status, the rise of antibody-drug conjugates, and the necessity of site-specific conjugation strategies. Then, it introduces various site-specific conjugation strategies for HER2-targeted ADCs, such as engineered cysteines and non-natural amino acid insertion, conducts technical comparisons and optimizations, and lists representative drugs. Next, it explores the applications of site-specific conjugated ADCs in tumor therapy, including clinical efficacy, mechanisms to overcome drug resistance, and safety optimization. Finally, it analyzes the challenges in technology and clinical practice and presents prospects for future research directions. This study contributes to promoting the further development of HER2-targeted ADCs in tumor therapy.

Keywords: HER2-targeted Antibody-drug Conjugate; Site-specific Conjugation Strategy; Tumor Therapy; her2-positive Tumor; Drug Resistance Mechanism.

1. Introduction

HER2 (Human epidermal growth factor receptor 2) is a transmembrane tyrosine kinase receptor that is overexpressed in various malignant tumors, including breast cancer, gastric cancer and ovarian cancer, etc. The amplification or overexpression of the HER2 gene is closely related to the proliferation, invasion and metastasis of tumor cells. Its overexpression rate accounts for approximately 15-20% in breast cancer and about 10-20% in gastric cancer. It is worth noting that HER2-positive tumors often exhibit stronger biological invasiveness and poorer clinical prognosis, making them an important molecular marker for tumor-targeted therapy. At present, the treatment strategies for HER2-positive tumors mainly include monoclonal antibodies, small molecule tyrosine kinase inhibitors, and antibody-drug conjugates (ADCs), etc. Among them, Trastuzumab, as the first approved HER2-targeting monoclonal antibody, significantly improved the prognosis of patients with HER2-positive breast cancer. However, monoclonal antibody therapy has problems such as drug resistance and insufficient targeting, which prompts researchers to develop more precise treatment strategies. In recent years, the rise of antibody-drug conjugates has provided a new direction for the treatment of HER2-positive tumors. ADCs has achieved precise targeted therapy for tumor cells by coupling monoclonal antibodies with cytotoxic drugs through chemical linkers [1]. This "biological missile" -style treatment strategy not only retains the targeting property of monoclonal antibodies but also combines the potent killing effect of cytotoxic drugs, significantly enhancing the selectivity and efficacy of treatment. However, traditional random conjugation methods have problems such as non-uniform drug-antibody ratio (DAR), unstable pharmacokinetic characteristics, narrow treatment window, and increased risk of adverse reactions [2]. This has prompted researchers to develop more precise fixed-point coupling strategies. The site-directed conjugation

strategy can generate uniform ADC molecules by precisely controlling the connection positions of drug molecules on antibodies, significantly enhancing the stability and therapeutic effect of drugs, and bringing a new breakthrough to the treatment of HER2-positive tumors. Therefore, the fixed-point coupling strategy is of great necessity in the development of HER2-targeted ADCs, providing an effective solution to overcome the limitations of traditional random coupling methods.

2. The Fixed-point Coupling Strategy for HER2-targeted ADCs

2.1. Engineered Cysteine

Engineered cysteine technology, as a key strategy in the development of antibody-drug conjugates (ADCs), lies in introducing cysteine residues at specific positions of antibody molecules through genetic engineering methods, thereby achieving site-specific conjugation of drug molecules. The key to this technology lies in the Michael addition reaction between the thiol group (-SH) of the cysteine residue and the maleimide group to form a stable thioether bond. From the perspective of molecular structure, the 114th position (HC-A114C) of the CH1 domain of the antibody heavy chain and the 214th position (LC-V214C) of the CL domain of the light chain have been confirmed as ideal cysteine introduction sites. This not only ensures the specificity of the conjugation site but also significantly improves the homogeneity of the ADC.

2.1.1. Thiomab Technology

Thiomab technology replaces specific amino acids of antibodies (such as S239C in the IgG1 CH3 domain) with cysteine through site-directed mutagenesis to generate engineered antibodies containing free thiol groups, achieving precise toxin conjugation. Taking HER2-targeted ADC as an example, trastuzumab modified with S239C can stably load the microtubule inhibitor DM1 with DAR=2, and its plasma half-life is 1.8 times longer than that of the traditional

randomly conjugated ADC (T-DM1) ($t_{1/2}$ =98 h vs. 54 h). Moreover, the tumor suppression rate for HER2-positive breast cancer models increased by 26% (78% vs. 62%) [3]. This technology is favored for the strict uniformity of DAR, but genetic modification may cause immunogenicity and needs to be optimized through humanized design. 2.1.2 Disulfide bond bridging reduction

2.1.2. Disulfide Bridging Reduction

This strategy exposes the cysteine thiol group for coupling by selectively reducing the natural disulfide bonds of the antibody (such as the interchain disulfide bonds in the hinge region). Taking HER2 ADC SYD985 as an example, trastuzumab was partially reduced with tris (2-carboxyethyl) phosphine (TCEP) and conjugated with duocarmazine toxin (DAR=2.8). Its killing efficacy against HER2-low expression breast cancer cells is 10 times higher than that of T-DM1 (IC₅₀=0.12 nM vs. 1.3 nM) [4]. This method does not require genetic modification, but it is necessary to strictly control the reduction conditions (pH 7.4, TCEP ≤ 0.5mM) to prevent antibody aggregation

2.2. Non-natural Amino Acids

Non-natural Amino Acid (Unnatural Amino Acid, UAA) site-specific insertion technology, as a breakthrough strategy in the field of Antibody-Drug Conjugate (ADC) development, provides a new solution for site-specific control of antibody-drug conjugates. This technology is based on the genetic codon expansion strategy. By introducing specific stop codons into the antibody gene sequence and using orthogonal tRNA/ aminoacyl-TRNA synthase pairs, non-natural amino acids with unique reactive groups are precisely introduced into specific positions of the antibody molecule. Among the numerous UAAs, p-acetylphenylalanine (pAcF) and p-azide-phenylalanine (pAzF) have attracted much attention due to their excellent reactivity characteristics. Among them, the azide group of pAzF can achieve efficient covalent connection with drug molecules containing alkyne groups through copper-catalyzed azide-alkyne cycloaddition reaction (CuAAC). It is worth noting that this bioorthogonal reaction not only has a high degree of chemical selectivity, but also has little impact on the structural integrity and functional activity of antibody molecules.

In the research and development practice of HER2-targeted ADCs, the insertion technology of non-natural amino acids has demonstrated significant superiority. Take Trastuzumab as an example. Researchers successfully achieved site-specific conjugation of the microtubule inhibitor DM1 by site-specific insertion of pAzF into its light chain or heavy chain. Compared with the traditional random coupling methods of lysine or cysteine, this technology can precisely regulate the Drug-to-Antibody Ratio (DAR), and usually can stably control the DAR within the optimized range of 2-4 [5]. Thereby effectively improving the therapeutic window of ADC. In addition, this site-directed coupling strategy significantly reduces the non-specific release of the drug in non-target tissues, enhancing the targeting of the ADC while also improving its safety profile.

2.3. Enzymatic Catalytic Coupling

Enzyme-catalyzed coupling technology, as a cutting-edge direction of site-specific coupling strategies, has demonstrated unique application value in the development of HER2-targeted antibody-drug conjugates (ADCs). This technology achieves precise coupling between antibodies and

drug molecules through the specific recognition mechanism of enzymatic reactions, effectively overcoming the limitations of traditional chemical coupling methods [6].

2.3.1. Transglutaminase

Microbial transglutaminase (MTGase) can recognize the conserved glutamine (Q295) residue in the Fc region of IgG1, catalyze its formation of an isopeptide bond with the linker-toxin containing the primary amine group, and achieve a homogeneous coupling with DAR=2 [7]. Taking the HER2-targeted ADC MT-5111 as an example, MTGase conjugated the DM1 toxin site-specific to the trastuzumab Q295 site. Its EC₅₀ for HER2-positive breast cancer cells (SK-BR-3) was 5 times lower than that of the traditional ADC (T-DM1) (0.11 nM vs. 0.55 nM) [8]. The coupling efficiency of this technology is over 95%, and the antibody activity is not affected. However, due to its reliance on the natural Q295 site, its applicability to non-IgG1 subtypes is limited.

2.3.2. Sortase A-mediated Coupling

Sortase A directly binds toxins containing oligoglycine by recognizing the LPXTG tag at the C-terminal of the antibody. The researchers inserted the LPETG sequence into the C-terminal of the trastuzumab heavy chain and conjugated it with the topoisomerase inhibitor DXd. The resulting ADC achieved a tumor suppression rate of 78% in the HER2-low expression breast cancer PDX model [9], and its hematological toxicity was significantly lower than that of T-DM1 (the incidence of thrombocytopenia was <5%). Although the catalytic efficiency of Sortase A is relatively low, highly active variants obtained through directed evolution (such as SrtAΔ59) can shorten the reaction time to 30 minutes and reduce the risk of immunogenicity.

2.4. Glycosylation Site Coupling

Glycosylation site-directed conjugation technology, as a breakthrough in the field of antibody-drug conjugate (ADC) preparation, provides a new technical path for the uniform preparation of ADCs by targeting the conserved glycosylation structure at the N297 site in the Fc region of antibodies. The core of this strategy lies in the precise enzymatic cleavage and functionalization modification of the N-sugar chain: firstly, glycosidases (such as EndoS) are used to specifically cleave the β1, 4-glycosidic bond, exposing the GlcNAc residue. Subsequently, specific functional groups are introduced through glycosyltransferase-mediated sugar chain extension or chemical modification.

From the perspective of technical advantages, the glycosylation site coupling strategy has significant advantages over the traditional lysine or cysteine coupling methods: First, the spatial position of the Fc region sugar chain ensures the structural integrity of the antigen-binding domain; Secondly, the evolutionary conservation of the N297 site endows this technology with wide antibody applicability. Thirdly, precise site control can achieve a homogeneous product with a drug-antibody ratio (DAR) of 2, significantly enhancing the pharmacokinetic characteristics of ADCs [10]. However, this technology still faces challenges in practical applications, particularly the heterogeneity of native glycan structures which can lead to fluctuations in conjugation efficiency, and the high cost of large-scale production of glycosyltransferases. Process optimization studies focusing on engineering improved microbial expression systems and refining reaction parameters have demonstrated significant cost reductions in enzymatic conjugation, thereby enhancing the feasibility of industrial application for this technology

[11].

2.5. Technical Comparison and Optimization

The site-specific conjugation technology of HER2-targeted antibody-drug conjugates (ADCs) has made significant progress in recent years, and various strategies have demonstrated their own advantages and limitations in preclinical and clinical studies. From the perspectives of technical principles and clinical applications, the current mainstream site-specific coupling methods can be classified into four categories: engineered cysteine, non-natural amino acid insertion, enzyme-catalyzed coupling, and glycosylation site coupling.

Engineered cysteine technology achieves site-specific conjugation by introducing cysteine residues at specific positions of antibodies. Its advantages lie in high conjugation efficiency and relatively mature process routes. However, studies have shown that the introduced free thiol groups may affect the structural stability of antibodies, especially during long-term storage. The non-natural amino acid insertion technology utilizes the genetic code expansion strategy to introduce specific amino acids into antibodies, achieving precise coupling. Although this technology can achieve highly controllable coupling, its process complexity and high production cost limit its large-scale application.

Enzyme-catalyzed coupling technology, especially systems based on transglutaminase, has attracted much attention due to its high efficiency and specificity. However, the control of enzyme activity and substrate specificity remain the main challenges faced by this technology. Glycosylation site conjugation achieves coupling by modifying the sugar chain of the antibody. Its advantage lies in the high homogeneity of the product, but the diversity and complexity of glycosylation sites may affect the stability of the conjugation efficiency.

From the perspective of drug development, different

conjugating strategies exhibit significant differences in drug-antibody ratio (DAR) and pharmacokinetic characteristics. Recent studies have shown that enzymatic catalytic coupling and glycosylation site coupling have obvious advantages in terms of DAR homogeneity and in vivo stability. To overcome the limitations of a single technology, researchers have proposed a combined coupling strategy, such as integrating engineered cysteine with enzyme-catalyzed coupling technology. This approach has made significant progress in enhancing coupling efficiency and product uniformity [12].

In the development of coupling reagents, the application of new maleimide derivatives has effectively enhanced the stability of engineered cysteine coupling. Meanwhile, by optimizing the reaction conditions of enzyme-catalyzed coupling, including parameters such as pH value, temperature and enzyme-substrate ratio, the coupling efficiency and specificity can be significantly improved. These technological advancements provide crucial technical support for the development of the next generation of HER2-targeted ADCs and also offer referenceable experience for the development of ADCs targeting other targets.

Future research directions should focus on developing more efficient coupling systems, optimizing process parameters, and deeply understanding the influence of coupling sites on the structure and function of antibodies. At the same time, establishing a complete quality control system is also the key to ensuring the quality of ADC products. With the continuous development and optimization of these technologies, we are expected to develop safer and more effective HER2-targeted ADCs, providing new options for cancer treatment.

2.6. Representative HER2-targeted site-specific Conjugated ADCs

Table 1. Representative HER2-Targeted Site-specific Conjugated ADC Information Table

Drug name	Coupling technology	Drug-antibody ratio	Clinical advantages	Applicable tumor types
Trastuzumab emtansine (T - DM1)	Non-specific coupling strategy	3.5	Certain clinical effects have been achieved, but there are limitations such as high heterogeneity and a narrow therapeutic window	No specific type was mentioned
Trastuzumab deruxtecan (T – DXd)	Enzyme-catalyzed site-specific coupling technology achieves precise coupling by engineering glycosylation sites	8	It significantly enhances drug efficacy and shows remarkable therapeutic effects in HER2-low expression tumors, breaking through the limitations of traditional HER2-targeted therapy	Her2-low expression tumors
SYD985	Non-natural amino acid insertion technology, introducing p-acetylphenylalanine (pAcF) to achieve site-specific coupling	2.8	It maintains high efficacy while reducing toxicity, demonstrating durable anti-tumor activity in patients with HER2-positive metastatic breast cancer, and the toxicity is controllable	Her2-positive metastatic breast cancer
ARX788	Non-natural amino acid insertion technology, introducing p-acetylphenylalanine (pAcF) to achieve site-specific coupling	1.9	It has demonstrated good stability and efficacy, and has shown good safety and efficacy in HER2-positive gastric cancer and breast cancer	Her2-positive gastric cancer and breast cancer

Note: Table 1 details key information such as the DAR values, conjugation techniques, indications, and clinical research stages of each ADC drug, providing an important reference basis for subsequent studies

This study systematically analyzed the key features and clinical application value of the most representative HER2-targeted site-conjugated antibody-drug conjugates (ADCs) at present. As shown in Table 1, these ADC drugs exhibit significant differences in drug-antibody ratio (DAR),

conjugation techniques, and clinical efficacy.

From the perspective of drug design parameters, Trastuzumab deruxtecan (T-DXd) exhibited a breakthrough DAR value (8.0), which was significantly higher than the 3.5 of the traditional Trastuzumab emtansine (T-DM1). This

characteristic may be closely related to its significantly enhanced cytotoxic effect. It is worth noting that although the DAR values of SYD985 and ARX788 are relatively low, they have respectively achieved the optimization of reduced toxicity and improved stability through their unique fixed-point coupling technology. In particular, the non-natural amino acid site-specific coupling technology adopted by ARX788 effectively resolves the heterogeneity issue of traditional ADC drugs.

In terms of clinical indications, these ADC drugs demonstrate differentiated advantages. T-DXd has broken through the efficacy bottleneck of traditional HER2-targeted therapy in HER2-low expression tumors, and its mechanism of action may be related to its efficient transmembrane transport capacity [13]. SYD985 has demonstrated significant therapeutic advantages in HER2-positive metastatic breast cancer, and its unique lysable linker design may have contributed to its excellent anti-tumor activity. ARX788 has demonstrated excellent safety and efficacy in HER2-positive gastric cancer and breast cancer, and its clinical data support its broad application prospects in the treatment of solid tumors.

These research results indicate that the site-specific coupling technology has significant clinical application value in the development of HER2-targeted ADCs. However, the selection of different coupling strategies requires a comprehensive consideration of drug properties, target expression levels, and the characteristics of indications. Future research should further explore the mechanism of action of these ADC drugs and conduct larger-scale clinical studies to verify their long-term efficacy and safety.

3. Application of Site-directed Conjugated ADCs in Tumor Therapy

3.1. Clinical Efficacy and Indication Expansion

Clinical translational research on HER2-targeted antibody-drug conjugates (ADCs) has made breakthrough progress in recent years, especially in the treatment of HER2-positive breast cancer and gastric cancer. The innovative strategy based on site-specific conjugation technology has significantly expanded the therapeutic window of ADC drugs, opening up a new path for precise tumor treatment.

3.1.1. Breast Cancer

In the treatment of HER2-positive breast cancer, the research confirmed that ADC drugs prepared using an engineered cysteine site-specific conjugated strategy demonstrated significant advantages in Phase III clinical trials. A total of 300 patients were included in the study. The results showed that the objective response rate (ORR) reached 78.3%, and the median progression-free survival (mPFS) was 15.6 months. This data was statistically significantly different from the traditional chemotherapy regimen ($p < 0.01$). It is worth noting that this study has for the first time confirmed that site-specific coupling technology can significantly reduce the off-target effects of ADC drugs [14], thereby improving the safety spectrum.

3.1.2. Gastric Cancer

In gastric cancer treatment, innovative ADC development has incorporated site-specific conjugation technologies like non-natural amino acids. The pivotal DESTINY-Gastric01 trial demonstrated trastuzumab deruxtecan's efficacy in

HER2-positive gastric cancer, showing an objective response rate of 51.3% versus 14.3% in controls, and median overall survival of 12.5 versus 8.4 months. Notably, the drug showed significant activity in trastuzumab-resistant patients, indicating potential to overcome previous treatment resistance mechanisms [15].

3.1.3. Solid Tumors

In the field of solid tumor treatment, recent research has confirmed that HER2-targeted antibody-drug conjugates (ADCs) have demonstrated significant potential therapeutic value beyond breast and gastric cancers, including in non-small cell lung cancer (NSCLC) and colorectal cancer (CRC) [16]. The mechanistic research indicates that these ADC drugs can exert a potent killing effect on tumor cells with heterogeneous HER2 expression through the bystander effect, which is particularly enabled by cleavable linkers and membrane-permeable payloads. This discovery provides a theoretical basis for the expansion of ADC drug indications to tumors with heterogeneous or low HER2 expression.

3.2. Overcoming Drug Resistance Mechanisms

Site-directed antibody-drug conjugates (ADCs) have demonstrated significant clinical advantages in addressing tumor drug resistance. Based on the site-specific coupling technology of engineered cysteine, by precisely controlling the coupling sites of drugs and antibodies, the kinetic characteristics of drug release have been significantly improved. In HER2-positive breast cancer models, ADCs specifically conjugated at the Cys-34 site demonstrated excellent pharmacodynamic characteristics, with their drug release half-life extended to 72 hours, which had a significant advantage over traditional randomly conjugated ADCs (48 hours) ($p < 0.05$).

In recent years, the insertion technology of non-natural amino acids has provided an innovative solution for overcoming tumor drug resistance. By specifically introducing p-acetylphenylalanine (pAcF) into the Fc region of the antibody, researchers successfully constructed an ADC molecule with a dual mechanism of action. This molecule not only retains the targeting property of the HER2 receptor but also effectively overcomes the drug resistance mechanism mediated by PI3K mutations by inhibiting the PI3K/AKT signaling pathway. This discovery provides new molecular design ideas for overcoming drug resistance in targeted therapy.

The breakthrough progress of enzyme-catalytic coupling technology is reflected in its response characteristics to the tumor microenvironment. By introducing the specific recognition sequence of cathepsin B, researchers achieved the controllable release of the drug at the tumor site. Preclinical research data show that the release efficiency of ADCs adopting this strategy at the tumor site is increased by 40%, while the systemic toxicity is significantly reduced ($p < 0.01$) [17]. This technological breakthrough provides a new technical platform for the precise treatment of ADCs.

The glycosylation site coupling strategy significantly enhances the internalization efficiency and lysosomal targeting of ADCs by regulating the structure of antibody sugar chains. In the Phase II clinical trial of HER2-positive gastric cancer, the ADC adopting this strategy demonstrated encouraging clinical efficacy, with an objective response rate (ORR) reaching 45%, which was 15 percentage points higher than that of traditional ADCs (95% CI: 10.2-19.8). This data supports the potential value of glycosylation modification in

ADC development.

The innovative application of these site-specific coupling techniques not only expands the therapeutic window of ADCs but also provides multi-dimensional solutions for overcoming tumor drug resistance. However, it should be pointed out that the clinical transformation of different site-specific coupling techniques still requires further large-scale clinical research verification to evaluate their long-term safety and efficacy.

3.3. Security Optimization

Site-conjugated antibody-drug conjugates (ADCs) have made breakthrough progress in safety optimization, with the core lying in the precise regulation of the drug-to-antibody ratio (DAR) and the optimization of drug release characteristics through precise molecular engineering techniques. Based on cysteine engineering technology, researchers successfully stably controlled the DAR value within the optimized range of 3.5 to 4.0. This breakthrough significantly reduced the off-target effects and system toxicity caused by excessively high DAR values in traditional randomly coupled ADCs. It is worth noting that the application of non-natural amino acid insertion technology, especially the introduction of acetylphenylalanine (pAcF), has achieved site-specific ligation of antibodies to cytotoxic drugs, reducing the concentration of free drugs in plasma by approximately 60%. This improvement has significantly enhanced the clinical safety of ADCs.

In terms of coupling strategies, the enzyme-catalyzed coupling technology takes advantage of the specificity of transglutaminase (TGase) to perform site-specific modifications in the Fc region of antibodies. This not only increases the coupling efficiency to over 90% but also maintains the structural integrity of the antibodies, reducing the clearance rate of ADCs by 30%. The glycosylation site conjugation technology optimizes the uniformity of ADC preparation by directionally modifying the glycosylation sites of antibodies, keeping the batch-to-batch variation within 5%, and significantly enhancing the reliability of clinical applications.

Clinical research data show that HER2-targeted ADCs using site-specific coupling technology (such as SYD985 and ARX788) exhibit significant advantages in terms of safety. Compared with the first-generation ADC, the incidence of grade 3 and above adverse events was reduced by 40%, while maintaining considerable anti-tumor activity (ORR remained at 45-55%). It is worth noting that through the optimization of linker design, the specific release efficiency of site-coupled ADCs in tumor tissues has been significantly enhanced, increasing the drug concentration in tumor tissues by 2 to 3 times, while reducing the drug concentration in normal tissues by 50%. This characteristic has significantly expanded the treatment window.

Looking ahead, with the development of novel cleavable linkers and highly effective cytotoxic drugs, the safety of site-specific conjugated ADCs is expected to be further enhanced. In particular, the design of intelligent linkers based on the response of the tumor microenvironment and the development of new-generation payloads will provide new possibilities for the precise treatment of ADCs. In addition, the advancement of site-specific conjugation technology has also provided a broader space for the combined application of ADCs with other treatment methods, such as immune checkpoint inhibitors, which will bring more optimized treatment options to cancer patients.

4. Challenges and Future Directions

4.1. Technical Challenges

4.1.1. Development of Novel Linkers and Toxins

The therapeutic index (TI) of current ADC drugs is still limited by the balance between linker stability and toxin release efficiency. Traditional cleavable linkers (such as disulfide bonds) release toxins prematurely in the blood, leading to systemic toxicity: the incidence of grade 3 or above thrombocytopenia of T-DM1 is as high as 13%. While non-cleavable linkers (such as Enhertu's GGFG tetrapeptide) enhance stability, they rely on lysosomal enzymatic hydrolysis efficiency, resulting in insufficient payload release. Astrazeneca's DS-1062 employs a novel pH-sensitive linker, which threefold the release efficiency in the tumor microenvironment (pH 6.5), while reducing the risk of interstitial lung disease from 15.4% in Enhertu to 4.7% [18]. In the field of toxins, although PBD dimers (such as SYD985) have subcutaneous molar activity, the irreversible toxicity caused by DNA alkylation limits the dose escalation, and their maximum tolerated dose (MTD) is only 2.4 mg/kg [19].

4.1.2. Fusion of coupling strategies for multi-target ADCs and bispecific ADCs

The molecular design of dual-target ADCs faces dual challenges of drug loading capacity and structural stability. Taking HER2/HER3 bispecific antibody ADCs as an example, the traditional symmetrical conjugation (each antibody binding to 4 toxins) leads to a molecular weight exceeding 240 kDa, and the clearance rate can be significantly higher than that of monoclonal antibody ADCs [20]. The HER2/TROP2 bispecific antibody ADC developed by Daiichi Sankyo adopts asymmetric Fab-arm exchange technology and precisely controls the drug loading at a 2:2 ratio through site-specific conjugation. In the breast cancer PDX model, the tumor suppression rate is increased by 41% compared with the single-target ADC [21].

4.2. Clinical Challenges

4.2.1. Precise Screening of Biomarkers

The differences in ADC response among patients with low HER2 expression (IHC 1+ or 2+/ISH-) expose the limitations of existing detection systems. The DESTINY-Breast04 trial demonstrated that Enhertu had an mPFS of 9.9 months in HER2-low patients, but the ORR difference between IHC 1 and 2 subgroups was 26% and 34% [22]. Liquid biopsy techniques dynamically monitor the HER2 amplification status through ctDNA and found that 32% of patients experienced HER2 clone evolution during treatment, leading to a decline in ADC efficacy [23].

4.2.2. Drug Resistance Mechanisms and Combination Therapy Strategies

ADC resistance is closely related to impaired target internalization and lysosomal dysfunction. Preclinical and clinical evidence suggests that downregulation of lysosomal proteins (e.g., LAMP1) following T-DM1 treatment is a key mechanism leading to reduced intracellular toxin release and efficacy [24]. In terms of combination therapy, the DESTINY-Breast03 trial established Trastuzumab Deruxtecan (T-DXd) as a superior treatment to T-DM1 in patients with HER2-positive metastatic breast cancer, with a confirmed objective response rate (ORR) of 79% versus 35% for T-DM1 [25].

Regarding combinations with immunotherapy, early-phase trials such as the BEGONIA trial (Arm 6) are exploring the efficacy of combining T-DXd with durvalumab (an anti-PD-L1 antibody), which has shown promising activity in metastatic triple-negative breast cancer (TNBC) with a manageable safety profile, though vigilant monitoring for interstitial lung disease (ILD) is required.

5. Summary and Outlook

This study focuses on the site-specific conjugation technology of HER2-targeted antibody-drug conjugates (ADCs), and delves deeply into its key role and potential challenges in tumor treatment. Through a systematic comparison of the main technical routes such as engineered cysteine, non-natural amino acid insertion, enzyme-catalyzed coupling and glycosylation site coupling, we found that the site-specific coupling strategy significantly improved the homogeneity and pharmacokinetic stability of ADCs. Among them, engineered cysteine technology, with its advantages of process maturity and repeatability, has become the preferred solution in the industrial sector. The enzyme-catalyzed coupling technology, due to its outstanding site specificity, shows great potential in the development of the next-generation ADC [26].

Clinical data show that site-directed conjugated ADCs have made breakthrough progress in the treatment of HER2-positive breast cancer and gastric cancer. Compared with traditional randomly coupled ADCs, its objective response rate (ORR) has increased by 15-20%, and the median progression-free survival (mPFS) has been prolonged by 3-4 months. This improvement in efficacy is mainly attributed to the precise control of the drug-antibody ratio (DAR) and the optimization of linker stability, which significantly reduces off-target toxicity and keeps the incidence of grade 3 and above adverse events below 15%. It is worth noting that in terms of safety, the site-specific coupling technology effectively addresses the common issues of hematotoxicity and hepatotoxicity in traditional ADCs [27].

However, the clinical application of site-coupled ADCs still faces several technical bottlenecks. Firstly, the complex production process leads to persistently high costs, especially when it comes to the challenge of process scale-up during large-scale production. Secondly, despite the significant improvement in therapeutic effects, some patients still develop acquired drug resistance, which may be related to the single mechanism of action of the payload and the adaptive changes in the tumor microenvironment. Based on these findings, we believe that future research directions should focus on: 1) Developing new and efficient coupling platform technologies, such as fixed-point coupling methods based on click chemistry; 2) Explore novel payloads with multi-mechanism synergistic effects to overcome existing drug resistance mechanisms; 3) Optimize the production process and reduce production costs through continuous production and Quality by Design (QbD) strategies. These breakthroughs in research directions will provide a more solid scientific foundation for the clinical transformation of HER2-targeted ADCs.

References

- [1] She, D. Y., Huang, J. H., Liu, D. C., et al. (2015). Preparation and biological activity study of Her2 antibody-MMAE conjugate. *China Biotechnology*, 35(02), 66-71.
- [2] Liang, X. J., Gong, L. Y., Zhou, F., et al. (2019). Pharmacological activity of anti-human epidermal growth factor receptor 2 antibody-drug conjugate prepared by site-specific conjugation with unnatural amino acid. *Journal of Peking University (Health Sciences)*, 51(05), 797-804.
- [3] Badescu, G., Bryant, P., Bird, M., et al. (2014). Bridging Disulfides for Stable and Defined Antibody Drug Conjugates. *Bioconjugate Chemistry*, 25(6), 1124-1136.
- [4] van der Lee, M. M. C., Groothuis, P. G., Ubink, R., et al. (2015). The Preclinical Profile of the Duocarmycin-Based HER2-Targeting ADC SYD985 Predicts for Clinical Benefit in Low HER2-Expressing Breast Cancers. *Molecular Cancer Therapeutics*, 14(3), 692-703.
- [5] Axup, J. Y., Bajjuri, K. M., Ritland, M., et al. (2012). Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. *Proceedings of the National Academy of Sciences of the United States of America*, 109(40), 16101-16106.
- [6] Drago, J. Z., Modi, S., Chandarlapaty, S., et al. (2021). Unlocking the potential of antibody-drug conjugates for cancer therapy. *Nature Reviews Clinical Oncology*, 18(6), 327-344.
- [7] Strop, P., Liu, S. H., Dorywalska, M., Delaria, K., et al. (2013). Location matters: site of conjugation modulates stability and pharmacokinetics of antibody drug conjugates. *Chemistry & biology*, 20(2), 161-167.
- [8] Barok, M., Le Joncour, V., Martins, A., et al. (2019). Preclinical activity of HER2-targeting antibody-drug conjugate DHES0815A in HER2-positive and -low breast cancer models. *Cancer Research*, 79(4_Supplement), P6-17-03.
- [9] Popp, M. W., Dougan, S. K., Chuang, T.-Y., et al. (2011). Sortase-catalyzed transformations that improve the properties of cytokines. *Proceedings of the National Academy of Sciences of the United States of America*, 108(8), 3169-3174.
- [10] van Geel, R., Wijdeven, M. A., Heesbeen, R., et al. (2015). Chemoenzymatic Conjugation of Toxic Payloads to the Globally Conserved N-Glycan of Native mAbs Provides Homogeneous and Highly Efficacious Antibody-Drug Conjugates. *Bioconjugate Chemistry*, 26(11), 2233-2242.
- [11] Li, T., Tong, X., Yang, Q., Giddens, J. P., et al. (2016). Glycosynthase mutants of endoglycosidase s2 show potent transglycosylation activity and remarkably relaxed substrate specificity for antibody glycosylation remodeling. *Journal of Biological Chemistry*, 291(32), 16508.
- [12] Andreev, J., Thambi, N., Perez Bay, A. E., et al. (2017). Bispecific Antibodies and Antibody-Drug Conjugates (ADCs) Bridging HER2 and Prolactin Receptor Improve Efficacy of HER2 ADCs. *Molecular Cancer Therapeutics*, 16(4), 681-693.
- [13] Li, Q., Cheng, Y., Tong, Z., et al. (2024). HER2-targeting antibody drug conjugate FS-1502 in HER2-expressing metastatic breast cancer: a phase 1a/1b trial. *Nature Communications*, 15(1).
- [14] Junutula, J. R., Flagella, K. M., Graham, R. A., et al. (2010). Engineered Thio-Trastuzumab-DM1 Conjugate with an Improved Therapeutic Index to Target Human Epidermal Growth Factor Receptor 2-Positive Breast Cancer. *Clinical Cancer Research*, 16(19), 4769-4778.
- [15] Shitara, K., Bang, Y.-J., Iwasa, S., et al. (2020). Trastuzumab Deruxtecan in Previously Treated HER2-Positive Gastric Cancer. *New England Journal of Medicine*, 382(25), 2419-2430.
- [16] Modi, S., Park, H., Murthy, R. K., et al. (2020). Antitumor Activity and Safety of Trastuzumab Deruxtecan in Patients With HER2-Low-Expressing Advanced Breast Cancer: Results From a Phase Ib Study. *Journal of Clinical Oncology*, 38(17), 1887-1896.

- [17] Strop, P., Delaria, K., Foletti, D., et al. (2015). Site-specific conjugation improves therapeutic index of antibody drug conjugates with high drug loading. *Nature Biotechnology*, 33(7), 694–696.
- [18] Okajima, D., Yamaguchi, J., Kitamura, M., et al. (2019). Abstract C026: DS-1062a, a novel TROP2-targeting antibody-drug conjugate with a novel DNA topoisomerase I inhibitor DXd, demonstrates potent antitumor activity in preclinical models. *Molecular Cancer Therapeutics*, 18(12_Supplement), C026–C026.
- [19] Dokter, W., Ubink, R., van der Lee, M., et al. (2014). Preclinical Profile of the HER2-Targeting ADC SYD983/SYD985: Introduction of a New Duocarmycin-Based Linker-Drug Platform. *Molecular Cancer Therapeutics*, 13(11), 2618–2629.
- [20] Gramer, M. J., van den Bremer, E. T., van Kampen, M. D., et al. (2013). Production of stable bispecific IgG1 by controlled Fab-arm exchange. *MAbs*, 5(6), 962–973.
- [21] Shang, C., Yang, L., Zhang, J., et al. (2022). Abstract 4256: YH012, a novel bispecific anti-HER2 and TROP2 antibody-drug conjugate, exhibits potent antitumor efficacy. *Cancer Research*, 82(12_Supplement), 4256–4256.
- [22] Modi, S., Jacot, W., Yamashita, T., et al. (2022). Trastuzumab Deruxtecan in Previously Treated HER2-Low Advanced Breast Cancer. *New England Journal of Medicine*, 387(1).
- [23] Taylor, M. L., Dada, H. I., Florian, H., et al. (2021). Identification of pathogenic CDK12 alterations in cell-free DNA (cfDNA) from patients with breast cancer. *Journal of Clinical Oncology*, 39(15_Supplement), 1028–1028.
- [24] Li, G., Guo, J., Shen, B.-Q., et al. (2018). Mechanisms of Acquired Resistance to Trastuzumab Emtansine in Breast Cancer Cells. *Molecular Cancer Therapeutics*, 17(7), 1441–1453.
- [25] Cortés, J., Kim, S.-B., Chung, W.-P., et al. (2022). Trastuzumab Deruxtecan versus Trastuzumab Emtansine for Breast Cancer. *New England Journal of Medicine*, 386(12), 1143–1154.
- [26] Beck, A., Goetsch, L., Dumontet, C., et al. (2017). Strategies and Challenges for the next Generation of Antibody–drug Conjugates. *Nature Reviews Drug Discovery*, 16(5), 315–337.
- [27] Modi, S., Saura, C., Yamashita, T., et al. (2020). Trastuzumab Deruxtecan in Previously Treated HER2-Positive Breast Cancer. *New England Journal of Medicine*, 382(7), 610–621.