Linker Chemistry for Tumor Microenvironment-Responsive Payload Release in ADCs

Yuhe Wang*

Senior High School Affiliated to University of Shanghai for Science & Technology, Shanghai, China

*Corresponding author: YohannWang1221@outlook.com

Abstract. Antibody drug conjugates (ADCs) represent a class of transformative anti-cancer therapeutic drugs, and linker components play an important role in balancing the stability of plasma and tumor specific payload release. This article analyzes the chemical design of ADC linkers and their responsive release strategies to the unique tumor microenvironment (TME). Firstly, the linkers are systematically classified into cleavable and non-cleavable types, with a focus on their different mechanisms. Further analysis is conducted on the subtypes of cleavable linkers, including pH sensitive, reduction sensitive, and enzyme cleavable variants. Detailed instructions are provided on how to cleave each linker under specific TME conditions, such as acidic or high glutathione environments. This article reveals the key trade-off between the powerful bystander effect provided by the cuttable joint and the superior system stability of the uncut joint through comparative analysis. In addition to the established design, the article also explores prospective linker technologies that respond to undeveloped TME cues such as hypoxia, elevated reactive oxygen species (ROS), and metal ions, and proposes new concepts such as dual reaction and gate linkers. This comprehensive analysis not only provides a framework for understanding current connector technology, but also offers valuable insights for guiding the rational design of next-generation ADCs with enhanced therapeutic indices. The continuous innovation of joint chemistry is crucial for realizing the full potential of precision oncology.

Keywords: Antibody-drug conjugates; tumor microenvironment; ADCs linkers.

1. Introduction

The concept of "selectively delivering toxic agents to disease-causing target cells" was first proposed by Paul Ehrlich in 1913—a vision that laid the foundation for targeted therapeutics and roughly 45 years later, materialized in the form of Antibody-Drug Conjugates (ADCs) [1]. ADCs operate on a precise mechanism: antibodies, which recognize tumor cells specifically, serve as "carriers" to deliver toxic agents directly to these targets, with the two components linked by chemical linkers [2]. This targeted design aims to maximize anticancer efficacy while minimizing damage to healthy cells, marking a pivotal advancement in oncology.

To date, ADC research has evolved through three distinct generations, each addressing limitations of the prior to enhance safety and efficacy. The first-generation ADCs, exemplified by BR96-doxorubicin, paired conventional chemotherapy drugs with mouse-derived antibodies via non-cleavable linkers. However, this iteration showed limited therapeutic effects, hindered by the suboptimal potency of cytotoxic agents and immunogenicity from mouse antibodies [3]. The subsequent shift to humanized monoclonal antibodies (mAbs) and more potent cytotoxic agents improved both efficacy and safety, yet challenges remained: short half-lives, rapid clearance, and residual immunogenicity still constrained clinical performance [3].

The second generation of ADCs brought comprehensive upgrades to all three core components—antibodies, toxic agents, and linkers—significantly boosting safety and efficiency. Nevertheless, unmet needs persisted, including off-target toxicity (due to imperfect linker stability) and drug aggregation, which limited their clinical application [3]. The third generation, represented by polatuzumab vedotin, enfortumab vedotin, and fam-trastuzumab deruxtecan, addressed these gaps: these ADCs exhibit lower toxicity, higher anticancer activity and enhanced stability, setting a new standard for targeted cancer therapy [3].

Notably, the advancement of linkers has played a critical role in driving ADC evolution—linker design directly impacts drug release specificity, stability in circulation, and ultimately therapeutic outcomes. Against this backdrop, this article focuses on the function of different linker types, conducts a comparative analysis of cleavable and non-cleavable linkers, and finally proposes novel ideas for designing next-generation linkers by leveraging previously untapped characteristics of the tumor microenvironment.

2. The Role and Classification of Antibody Drug Conjugates (ADC) Linkers

The linker of antibody drug conjugates (ADCs) plays an indispensable and critical role throughout their entire process of action. The linker first needs to maintain sufficient stability in the plasma environment to prevent premature release of cytotoxic drugs - once premature release occurs, it may cause damage to healthy cells and even lead to their death. At the same time, when ADC undergoes endocytosis with target cells, the linker must be able to release toxic substances in a timely manner to ensure precise drug delivery to tumor cells. Based on such functional requirements, ADC connectors are currently mainly divided into two categories: cleavable connectors and non-cleavable connectors. These two types of connectors have their own characteristics in structural design and mechanism of action, and are suitable for different treatment scenarios.

3. Cutable Connectors

The core mechanism of cleavable linkers is to break through the tumor microenvironment, the environmental differences between tumor cell endosomes and plasma, and release cytotoxic drugs. Common cleavable linkers mainly include chemically unstable linkers and enzyme-sensitive linkers, among which chemically unstable linkers can be further divided into pH-sensitive, reduction-sensitive and oxidation-sensitive linkers. These different types of linkers each respond to specific environmental signals to achieve targeted drug release [4].

3.1. PH-Sensitive Linkers

PH-sensitive linkers can maintain a stable structure in alkaline environments, but can break in acidic environments. These types of linkers typically contain hydrazone bonds, which undergo hydrolysis under acidic conditions, releasing cytotoxic drugs [4]. The extracellular pH value of normal tissues is about 7.4, which is in a relatively alkaline state, while the pH value of the tumor microenvironment is even lower, usually between 6.7-7.1. More importantly, the pH values of the endosomes (pH about 5-6) and lysosomes (pH about 4.8) inside tumor cells will further decrease [5]. Therefore, when ADC reaches the tumor environment or is internalized by tumor cells, the surrounding acidic environment will promote the cleavage of the hydrazone bond and release the drug. However, this type of linker is one of the least stable and may break prematurely in the bloodstream due to small changes in the local microenvironment, causing damage to healthy cells. At the same time, it also has a unique "bystander killing effect", which not only kills directly targeted tumor cells but also has a killing effect on nearby tumor cells, which has certain advantages in the scenario of tumor cell spread.

3.2. Reduction-Sensitive Linkers

The molecular structure of reduction-sensitive linkers typically contains disulfide bonds, and the cleavage of disulfide bonds depends on the reducing environment [4]. Intracellular glutathione is a tripeptide reducing agent that can break disulfide bonds through reduction [4,6]. The concentration of glutathione in the cytoplasm and nucleus (1-10 mM) is much higher than in the extracellular space and blood (2-20 μM). Especially in tumor cells, due to the need to combat oxidative stress, the expression level of glutathione is often higher. This huge concentration gradient (3-4 orders of magnitude difference) drives the selective reduction and cleavage of intracellular disulfide bonds,

thereby releasing drug payloads. In addition, enzymes such as protein disulfide isomerase (PDI) on the cell surface may also participate in extracellular reduction processes, but this is relatively rare and mainly depends on the high concentration of glutathione environment inside the cell.

3.3. Enzyme-Cleavable Linkers

Enzyme-cleavable linkers are the most common and successful type in modern ADC design, and their high specificity is mainly due to the unique enzyme spectrum of the tumor microenvironment (TME) and lysosome compartments. Its mechanism of action is to utilize the characteristic that the expression level of specific enzymes in tumor tissue is much higher than that in healthy tissue, in order to achieve targeted release of the drug load. Among them, the peptide linker contains short dipeptide sequences designed as optimal substrates for lysosomal proteases, such as the Val-Cit dipeptide, which is the most famous example. It is mainly cleaved by protease B, a cysteine protease that is highly expressed, secreted, and overactive in various human tumors [7]. In order to ensure efficient release of unmodified cytotoxic payloads, these peptide linkers are almost always paired with autophagic spacers (such as PABC). When the peptide is cleaved by enzymes, the PABC spacer rapidly and spontaneously undergoes a 1,6-elimination reaction, ultimately releasing the free active drug. Although the effect is significant, there is a key design challenge for such linkers, which is the need to optimize peptide sequences to achieve ultra-high stability in plasma, as circulating proteases such as carboxyesterase 1C (CES1C) sometimes exhibit low-level activity against certain peptides, which may lead to premature cleavage and systemic toxicity of the linkers. In addition to Val Kit, other sequences such as phenylalanine lysine (Phe Lys) are also widely used, and they are easily cleaved by proteases and other proteases [4].

The βglucuronide linker uses the hydrophilic glucuronic acid moiety as the cleavage site, and glucuronic acid is the specific substrate of β-glucuronidase. The concentration of β-glucuronidase increases in the necrotic core of many solid tumors because necrotic cells release this enzyme, which also has high activity in the lysosome. The main advantages of this type of linker are reflected in two aspects: firstly, its inherent hydrophilicity can significantly improve the solubility of ADC conjugates, effectively alleviate high hydrophobic loading and aggregation problems that often occur with peptide linkers, and ensure uniform distribution of drugs in the body; Secondly, its bulky glucuronic acid structure makes it an inefficient substrate for P-glycoprotein (P-gp) and other multidrug resistance (MDR) efflux pumps, which may have advantages over certain peptide linkers and help overcome the main mechanism of cancer cell chemotherapy resistance, thereby enhancing the efficacy of ADC.

Phosphatases can cleave linkers designed with phosphate groups as the core, which are substrates of alkaline phosphatase (ALP). Alkaline phosphatase is a recognized biomarker with significantly increased activity on the surface of various cancer cells (such as ovarian cancer, lung cancer, liver cancer cells) and in the tumor microenvironment, and is often associated with invasive and metastatic diseases of tumors. The main strategic advantage of this type of linker is its potential extracellular activation ability. Unlike lysosome-targeted linkers (which require ADC endocytosis to function), phosphate-based linkers can be directly cleaved by alkaline phosphatase on the cell surface, releasing a membrane-permeable payload and exerting a strong bystander effect on neighboring cells, regardless of their antigen expression or endocytosis efficiency. However, it also presents a significant challenge, as the concentration of alkaline phosphatase is relatively low in certain tumor environments, while phosphatase is commonly present in normal tissues such as the liver and bone. This requires careful stratification of patients based on the expression level of alkaline phosphatase to avoid damage to normal tissues.

4. Non-cleavable Connectors

There is a fundamental difference in design philosophy between non-cleavable linkers and cleavable linkers, as they lack specific cleavage sites. Their core design philosophy is to ensure absolute stability during systemic circulation and avoid premature release of drug loads. The release

of drug payloads from non-cleavable linkers relies entirely on the complete protein hydrolysis and degradation process of the antibody portion within the lysosome. During this process, the antibody is gradually broken down, typically resulting in the drug payload still being bound to and released from an amino acid residue (such as cysteine thiol or lysine side chain produced by antibody catabolism). Therefore, this' load amino acid complex 'must retain potent cytotoxic activity, otherwise, ADC cannot exert effective therapeutic effects. Although this design avoids the risk of premature release, it also limits its application in heterogeneous tumors as it cannot generate bystander effects[4].

5. Comparative Analysis of Connectives

The selection of cleavable and non-cleavable linkers is a crucial strategic decision that profoundly affects the pharmacological characteristics and therapeutic efficacy of ADC. The most significant functional difference between the two lies in their ability to mediate bystander killing effects, which directly determines their applicability in the treatment of different types of tumors. The cleavable linker is designed to release a free, unmodified drug payload that, if sufficiently hydrophobic and membrane permeable (such as monomethyl auristatin E, MMAE), can diffuse from dead target cells and enter adjacent cells. This characteristic is crucial for treating solid tumors, as solid tumors are often highly heterogeneous, containing a mixed population of antigen-positive and antigen-negative cancer cells, and bystander effects can effectively cover those antigen-negative tumor cells. In contrast, the cytotoxic substances released by uncut linkers are charged "load linker amino acid adducts", which typically cannot penetrate the cell membrane. This strictly limits the cytotoxic effects to antigen-positive cells that have internalized ADCs, making their therapeutic efficacy for heterogeneous tumors poorer. However, it may also reduce damage to off-target tissues and improve the safety of treatment.

This functional difference is directly related to the key parameter of systemic stability. The mechanism by which cleavable linkers can effectively release drugs in the tumor microenvironment also carries the risk of premature activation in the blood: for example, the hydrazone bond may undergo slow hydrolysis at neutral pH, the disulfide bond may be reduced by extracellular glutathione or serum proteins, and the peptide linker may be cleaved by circulating proteases. This premature release is the main cause of dose-limiting toxicity, such as neutropenia, which may significantly narrow the treatment window and limit the dosage of drugs used. The non-cleavable linker is composed of stable chemical bonds such as thioether, which has excellent plasma stability. This inertness in circulation can reduce off-target toxicity and is a key factor in broadening the therapeutic window, making it more advantageous in terms of safety.

Therefore, the choice of linker often determines the target indication of ADC. Due to its bystander effect, cleavable linkers are highly suitable for the treatment of solid tumors, as overcoming heterogeneity is the key to successful treatment in solid tumors; Non cleavable linkers often play an efficient role in hematological tumors because the target antigen of hematological tumors is expressed more evenly on all tumor cells, and cancer cells are easily accessible in the blood, without relying on bystander effects to achieve effective killing. This has also been reflected in clinical practice. For example, ADC based on non-cleavable linkers (such as Trastuzumab Metaxine conjugate, T-DM1) is used to treat HER2-positive breast cancer (a solid tumor), but its effectiveness also depends on the high antigen density and efficient endocytosis of tumor cells. It is worth noting that the design of modern ADCs is blurring these traditional classifications. For example, trastuzumab deruxtecan (T-DXd) uses cleavable tetrapeptide linkers, but due to its good membrane permeability and strong bystander effect, it embodies advanced hybrid design concepts and further expands the application boundaries of linkers.

6. Design Prospects of New Connectors

The development of ADC connectors is advancing towards utilizing more subtle and specific features of the tumor microenvironment, with the aim of achieving unprecedented selectivity, further improving therapeutic efficacy, and reducing toxicity. Among them, hypoxia-responsive linkers are an important research direction. Many solid tumors have severe hypoxia in their core regions[8]. To address this characteristic, linkers containing nitroaromatic or quinone motifs can be designed. These functional groups can be reduced by NAD (P) H: quinone oxidoreductase (NQO1), which is often highly expressed in hypoxic cancer cells, leading to linker fragmentation and release of drug payloads. This design can achieve targeted clearance of the most resistant tumor areas, improving the thoroughness of treatment.

Reactive oxygen species (ROS) sensitive linkers are also a highly promising research field. The high metabolic activity of tumor cells produces high levels of reactive oxygen species (such as hydrogen peroxide, H_2O_2) [9]. Based on this characteristic, linkers based on arylboronic esters or thioketones are designed, which undergo selective cleavage in the presence of ROS. This strategy can achieve rapid release of tumor microenvironment specificity while minimizing off-target activation in normal tissues, improving the accuracy of treatment.

The metal ion-catalyzed cleavage linker focuses on the imbalance of metal ion homeostasis in the tumor microenvironment, such as changes in the concentration of metal ions, such as copper (II) and zinc (II) [10]. Designing linkers that can coordinate with these specific ions can promote metal-catalyzed hydrolysis reactions within tumors, such as the hydrolysis of carboxylic esters. This design provides a novel and externally adjustable release mechanism, opening up new avenues for controlled release of ADCs.

The dual response and "AND gate" connectors represent the future direction of ADC specificity research, and their core idea is to use a combination triggering system, where the connector requires the simultaneous presence of two stimuli to be activated. For example, a linker may require simultaneous reduction (disulfide bond cleavage) and acidification (hydrazone bond hydrolysis) to release drug loading. This "AND gate" logic ensures that drug loading is only released in cells that simultaneously possess a specific combination of tumor microenvironment biomarkers, greatly improving the safety of treatment by preventing activation in healthy tissues that only have one condition. This design concept represents the next frontier for ADC to achieve a true maximum therapeutic window, and is expected to drive further development of ADC drugs.

7. Conclusion

In conclusion, the strategic design of the linker is a cornerstone in the development of effective and safe antibody-drug conjugates, dictating their usability by mediating the critical balance between systemic stability and tumor-specific cytotoxicity. This review has systematically delineated the fundamental mechanisms of cleavable and non-cleavable linkers, highlighting how their different designs lead to different relative merits. We have further explored the subclasses of cleavable linkers—pH-sensitive, reduction-sensitive, and enzyme-cleavable—each engineered to exploit a specific pathological hallmark of the tumor microenvironment, such as acidity, elevated glutathione, or overexpression of proteases like cathepsin B and β-glucuronidase. A comparative analysis underscored the inherent trade-offs: cleavable linkers facilitate a potent bystander effect crucial for treating heterogeneous tumors but may succumb to premature release and systemic toxicity, whereas non-cleavable linkers offer exceptional plasma stability and a narrower therapeutic effect confined to antigen-positive cells. Looking beyond established paradigms, we proposed innovative linker strategies responsive to underexplored TME conditions, including hypoxia, elevated reactive oxygen species, and metal ions, with pioneering concepts like dual-responsive AND-gate linkers representing the next frontier in precision targeting.

The insights garnered from this analysis provide a vital framework for rational linker selection and design, directly contributing to the advancement of ADC technology with the goal of achieving

a wider therapeutic index. However, several limitations persist. The heterogeneity of the TME across different cancer types and individual patients means a universal linker strategy is improbable; a one-size-fits-all approach may not exist. Furthermore, our understanding of extracellular cleavage kinetics and the potential for offtarget activation in benign tissues with inflammatory microenvironments remains incomplete. The translational gap between promising in vitro linker designs and their performance in complex in vivo models also presents a significant challenge.

Future outlooks must therefore prioritize the development of patient stratification biomarkers to match specific linker-payload constructs to the appropriate TME. Research should focus on engineering novel adaptive linkers that require multiple simultaneous stimuli for activation, thereby maximizing specificity. Finally, leveraging advanced conjugation techniques for site-specific attachment of these next-generation linkers will be essential to produce homogeneous ADC formulations with optimized pharmacokinetics. Ultimately, the continuous innovation in linker chemistry will be indispensable in unlocking the full potential of ADCs and solidifying their role in the future of precision oncology.

References

[1] Tsuchikama, K., & An, Z. (2018). Antibody-drug conjugates: recent advances in conjugation and linker chemistries. Protein & cell, 9(1), 33–46. https://doi.org/10.1007/s13238-016-0323-0

[2] Jin, Y., Schladetsch, M. A., Huang, X., Balunas, M. J., & Wiemer, A. J. (2022). Stepping forward in antibody-drug conjugate development. Pharmacology & therapeutics, 229, 107917. https://doi.org/10.1016/j.pharmthera.2021.107917

[3] Fu, Z., Li, S., Han, S., Shi, C., & Zhang, Y. (2022). Antibody drug conjugate: the "biological missile" for targeted cancer therapy. Signal transduction and targeted therapy, 7(1), 93. https://doi.org/10.1038/s41392-022-00947-7

[4] Sheyi, R., de la Torre, B. G., & Albericio, F. (2022). Linkers: An Assurance for Controlled Delivery of Antibody-Drug Conjugate. Pharmaceutics, 14(2), 396. https://doi.org/10.3390/pharmaceutics14020396

[5] Arneth B. (2019). Tumor Microenvironment. Medicina (Kaunas, Lithuania), 56(1), 15. https://doi.org/10.3390/medicina56010015

[6] Kalinina E. (2024). Glutathione-Dependent Pathways in Cancer Cells. International journal of molecular sciences, 25(15), 8423. https://doi.org/10.3390/ijms25158423

[7] Aggarwal, N., & Sloane, B. F. (2014). Cathepsin B: multiple roles in cancer. Proteomics. Clinical applications, 8(5-6), 427–437. https://doi.org/10.1002/prca.201300105

[8] Jing, X., Yang, F., Shao, C., Wei, K., Xie, M., Shen, H., & Shu, Y. (2019). Role of hypoxia in cancer therapy by regulating the tumor microenvironment. Molecular cancer, 18(1), 157. https://doi.org/10.1186/s12943-019-1089-9

[9] Nakamura, H., & Takada, K. (2021). Reactive oxygen species in cancer: Current findings and future directions. Cancer science, 112(10), 3945–3952. https://doi.org/10.1111/cas.15068

[10] Gao, Y., Liu, S., Huang, Y., Li, F., & Zhang, Y. (2024). Regulation of anti-tumor immunity by metal ion in the tumor microenvironment. Frontiers in immunology, 15, 1379365. https://doi.org/10.3389/fimmu.2024.1379365