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REVIEW

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Recent progress of enzyme cleavable linker in antibody–drug conjugates: sulfatase and phosphatase

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ABSTRACT

Recently, antibody–drug conjugates (ADCs) are used to deliver efficient cytotoxic payloads selectively in cancer cells. In the designing of an ADC, the antibody is connected to a toxic payload via a covalent linker, which helps to solubilizes the typical hydrophobic payload as well as stabilizes the linkage over circulation. The development of the linkers for the antibody drug conjugate is still in demand. Initially, the acid, disulfide, and cathepsin-sensitive ADCs attracted considerable attention for the delivery of a potent cytotoxic payload but suffer from instability in human and mouse plasma with a short half-life. In addition, It also suffer from a solubility issue that induces aggregation, which is the major problem in their development. ADCs associated with sulfatase and phosphatase cleavable linker are highly soluble due to the anionic nature of sulfate and phosphate groups. The ADCs also showed high stability in human and mouse plasma. Therefore, to overcome these limitations, sulfatase and phosphatase cleavable linkers were developed. This review focuses on the recently reported advantages of sulfatase and phosphatase cleavable linkers for ADCs.

Key Word: Cleavable linker, Antibody-drug conjugate, Sulfatase, Phosphatase

Introduction

Recently, antibody–drug conjugates (ADCs) therapeutic techniques are being developed for cancer treatment [1]. To date, there are nine food and drug administration-approved ADC medicines, whereas more than 80 are being studied [2]. An ADC comprises three components—an antibody, a linker (cleavable or noncleavable), and a toxic payload. The antibody provides cell selectivity and recognizes antigens with less or no countenance on healthy cells. The bonding between the antibody and linker expresses the drug-to-antibody ratio (DAR). In the ADC, the linker would be the easily modifiable part and play important role providing high plasma stability, water solubility, and long circulatory lifetimes. Importantly, it also avoids off-target payload release and ADC aggregation [3].

Initially, acid sensitive cleavable linker were developed for the successful delivery of drug in targeted cells. The purpose of development of acid cleavable linker was to utilize the acidity of lysosomes (pH 4.5 – 5.0) and endosomes (pH 5.5 – 6.2). But this linker suffered with instability in plasma and leading to premature release of drug in plasma circulation. Further, the reducible disulfide linker was susceptible to nucleophilic attack by thiols [4, 5]. To overcome with

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these limitaions, enzyme cleavable linker are used for the development of ADCs. Enzyme cleavable ADC linkers included protease [6], glycosidase [7], sulfatase cleavable motifs [8, 9], pyrophosphatase [10], and acid phosphatase [11]. At present, most ADCs which are under clinical trials are based on cathepsin-cleavable dipeptides. The ADCs containing dipeptide linkage tends to show similar shortcomings such as mouse plasma instability and ADC aggregation caused due the the hydrophobic nature of dipeptides, limiting the preclinical evaluation [12]. Despite this limitation, dipeptides are still employed in the development of ADC for the delivery of pyrrolobenzodiazepine dimer and monomethyl auristatin E (MMAE) payloads. Further, some nonpeptidic linkers containing ADCs, which are susceptible to cleavage with lysosomal β-galactosidase and β-glucuronidase enzymes, have been developed. These ADCs mitigate the problem associated with mouse plasma instability and linker payload hydrophobicity [13, 14]. However, their stereochemical complication and craving on other lysosomal enzymes may reduce the expression of ADCs for clinical studies. Overall, to overcome these common limitations, the attention of researchers has been paid to develope novel phosphatase and sulfatase enzyme based cleavable linkers which are proved to be effective in both human and rat plasma, increasing the stability and solubility.

Sulfatase cleavable linkers

Sulfatases are enzymes involved in the cleavage of the sulfate ester bond in biological systems; they play an essential role in monitoring the functions of many physiological molecules. They are concerned with many biological processes, such as bacterial pathogenesis, cell signaling development, and hormone regulation [15]. Moreover, Sulfatases are overexpressed in several types of cancers cells that give additional selectivity for cancer targeting ADCs [16]. Further, the sulfatase cleavable linker exhibit high stability in human and mouse plasma, but is reactive within a lysosomes that offer an additional opportunity for arylsulfate-containing ADCs toward a tumor for selective release of payloads (Figure 1).

In 2020, Bargh *et al.* used a sulfatase-based cleavable linker to develop a new class of ADCs [8]. The sulfatase cleavable linker were found highly soluble in water and showed high stability in human and mouse plasma. This linker exhibited high reactivity within the lysosomes.

Further, the stability of the linker was tested under

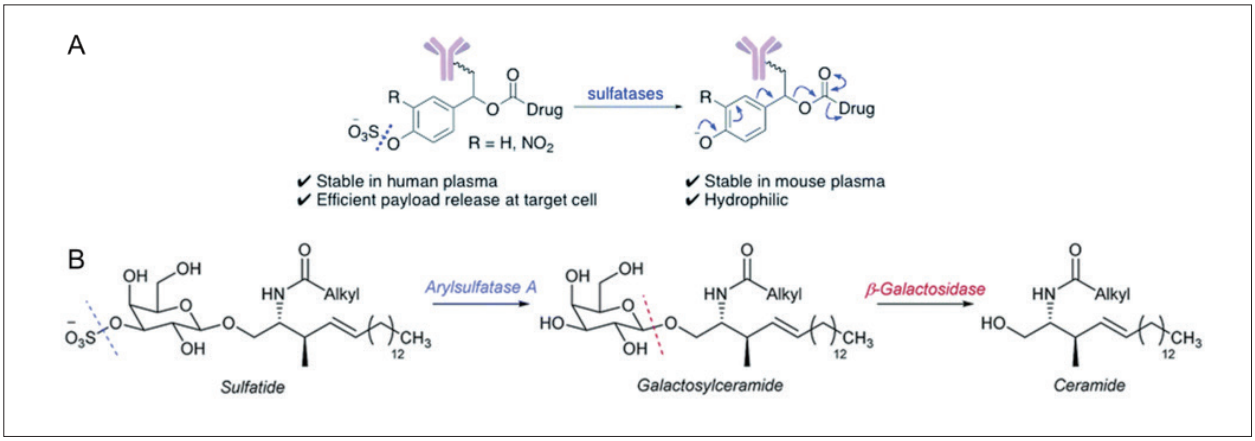

Figure 1. Model presentation for (A) arylsulfatase-based cleavable linker; (B) sulfatase and galactosidase-based cleavable linker. Reproduced from [8] and [9].

typical condition of the lysosomal compartment and blood plasma. The two different linkers, ortho amide-linked arylsulfate (ADC 1) and a benzyl-linked arylsulfate (ADC 2)—underwent 1, 6-elimination after interaction with sulfatase to release the payload. The rate of hydrolysis by sulfatase was found to be superior for benzyl linked arylsulfatase cleavable linker ($t_{1/2}$ = 24 min) compared with orthoamide linked arylsulfatase cleavable linker ($t_{1/2}$ > 12 h) under similar conditions. Therefore, the benzyl linked ADC would release the payload more efficiently when compared to ortho amide linker. Further, when the both the linkers were tested for stability in human and mouse plasma, found stable upto 7 days. However, other dipeptide linkers (ADC 3 and 5) underwent fast hydrolysis in mouse plasma ($t_{1/2}$ < 1 h). The linker payload were conjugated to transtuzumab to achieved ADC 1–5 (Figure 2A). The cytotoxicity of all ADCs was examined for HER2-positive (BT474) and HER2-negative (MCF7) cells (Figure 2B, and C). ADC 5—a cleavable dipeptide linker-based ADC—showed high cytotoxicity for the HER2-positive (BT474) cell, whereas ADC 4—the non-cleavable dipeptide linker-based ADC—was nontoxic (Figure 2B). However, ADC 2 and 3—benzyl-linked arylsulfate ADCs—showed cytotoxicity analogous to the dipeptide linker-based ADC; thus, the linker sensitive to arylsulfatase enzyme could also deliver payloads in cells followed by ADC internalization. Moreover, ADC 1 exhibited a nontoxic nature for HER2-positive cells. The nontoxic nature of ADC 1 revealed that, *in vitro*, the rate of cleavage of arylsulfate linker is insufficient and the anionic nature of the sulfate group near MMAE payloads restricts tubulin-binding affinity. However, all the five ADC 1–5 showed non toxic nature towards HER2-negative MCF7 cells (Figure 2C).

In 2021, the same group used a new dual-enzyme cleavable linker susceptible to arylsulfatase A and β-galactosidase for developing ADCs [9]. They employed a 3-O-sulfo-β-galactose linker to develop ADCs; this linker was sequentially cleaved by two different enzymes—arylsulfatase A and β-galactosidase—to deliver the potent payload in targeted cells. These two functional groups, anionic sulfate and pyranose, displayed high hydrophilicity that reduced the possibility of aggregation of ADC. The extracellular stability of the 3-O-sulfo-β-galactose linker was tested in human

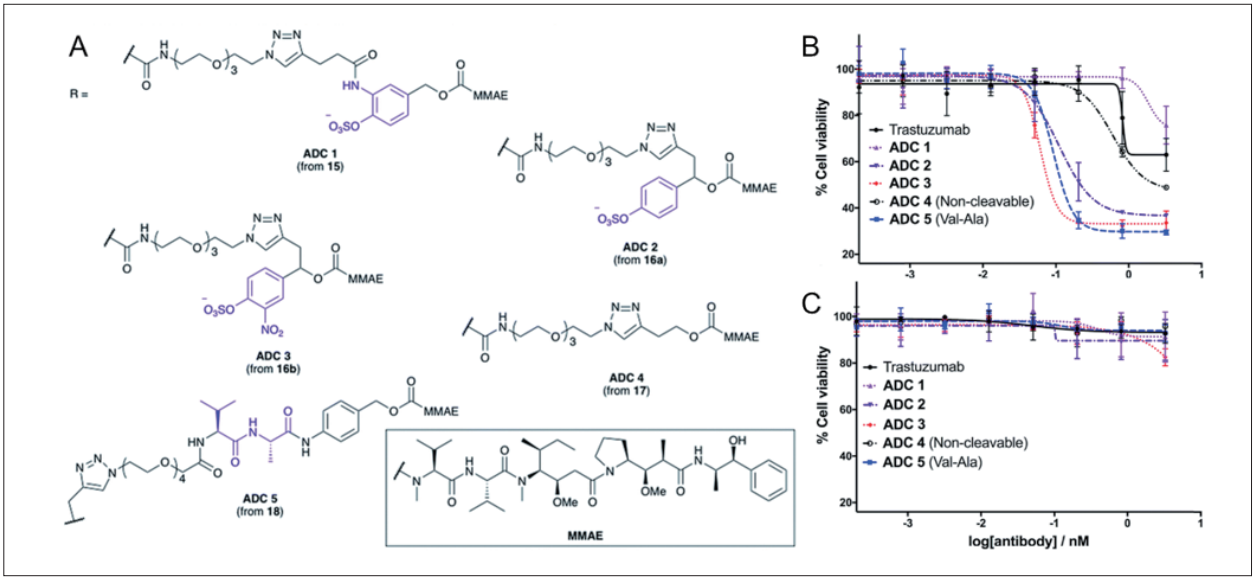

Figure 2. (A) Antibody–drug conjugates ADC 1–5. In vitro biological evaluation of ADC 1–5 in (B) HER2-positive BT474 cells and (C) HER2-negative cells. Reproduced from [8].

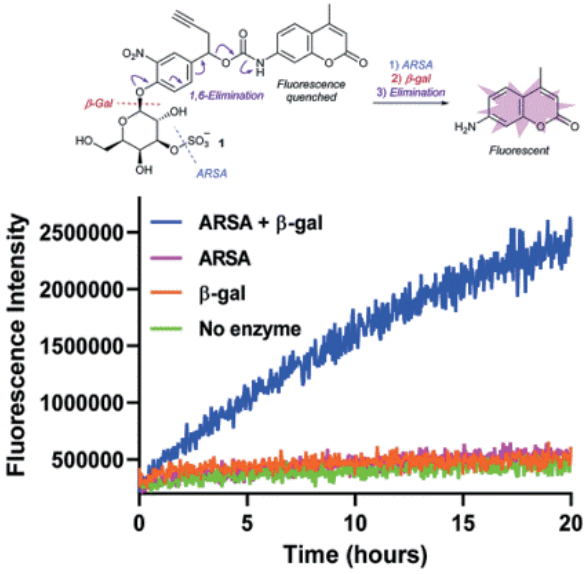

Figure 3. Releasing pattern of 7-amino-4-methyl coumarin after incubation with arylsulfatase and β-gal enzymes. λ_{ex} = 350 nm, λ_{em} = 460 nm (Fluorometric response). Reproduced from [9].

and mouse plasma, and found stable for more than 20 h. Further, this linker was found sensitive with both the enzymes simultaneously, whereas individual enzymes arylsulfatase A and β-galactosidase did not participate in cleavage of the linker (Figure 3). This result proposed the dual-enzyme catalysis nature of the 3-O-sulfo-β-galactose linked ADC for hydrolysis.

Further, the 3-O-sulfo-galactose linked ADC showed excessive cytotoxicity for HER2-positive SKBR3 cells, which is comparable to arylsulfate ADC analog [8], as mentioned above.

Phosphatase cleavable linkers

Phosphatases (pyrophosphatase and acid phosphatase) are another class of enzymes engaged in the cleavage of pyrophosphate and terminal monophosphate to their parent alcohols, respectively, in a biological medium. Phosphatases are of interest in pharmaceutical research because of their importance in cellular signaling and activity regulation [17]. The pyrophosphate linker is highly soluble in water, which facilitates the aqueous bioconjugation to antibodies and reduced the possibility of aggregation of ADC. In 2016, Kern *et al.* developed a series of phosphate diester linker (monophosphate, diphosphate, and triphosphate diester) under the assumption that if the drug linkers exhibited high stability in blood and showed high reactivity in the lysosomal environment, then they will retain their properties after bioconjugation with antibody [10]. They linked all phosphate diester series with dexamethasone payload and found all phosphate diester were stable in human and mouse plasma for more than 6 h. However, the releasing rate of dexamethasone payload with monophosphate in lysosomal extracts was sluggish compared with pyrophosphate and triphosphate diester. A similar result was observed with the budesonide payload, but that of fluticasone propionate was poor due to its bulky nature. When the bulkier fluticasone payload was modified with acetal spacer, its releasing rate increased and was found similar to releasing rate of dexamethasone and budesonide pyrophosphate linker in the lysosomal lysate (Figure 4A).

The ADCs bearing two linker payload, budesonide pyrophosphate and acetal fluticasone pyrophosphate diester were found stable in human and mouse plasma, no loss of budesonide and fluticasone observed up to 7 days. Further, these ADCs were incubated in lysosomal lysates for 6 h. The budesonide ADC showed the release of budesonide monophosphate first and then rapidly degraded to free budesonide (Figure 4B). Similarly, the fluticasone ADC first released fluticasone propionate acetal monophosphate and then rapidly changed to fluticasone propionate (Figure 4C).

In 2016, the same group established a new strategy for developing ADCs by employing a terminal monophosphate and pyrophosphate cleavable linker in a well-established cathepsin-cleavable dipeptide

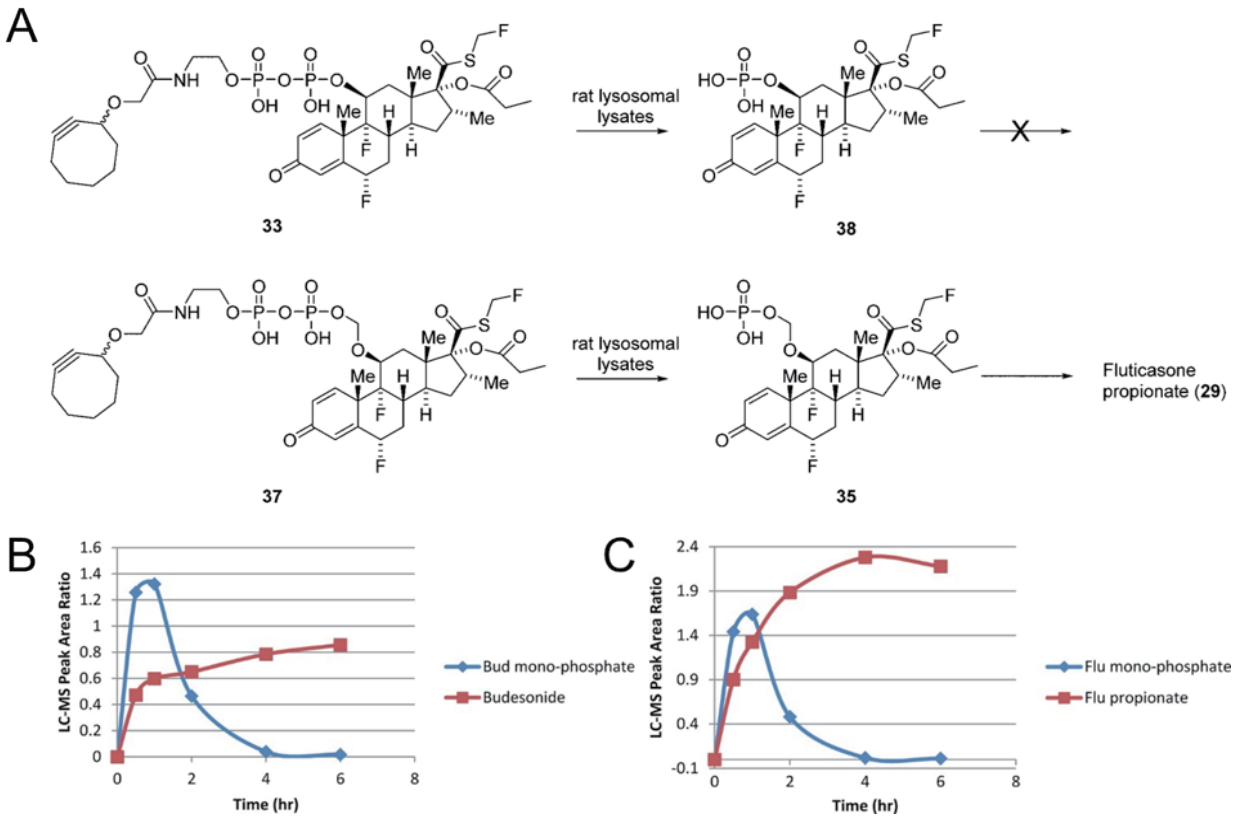

Figure 4. (A) Conversion of fluticasone propionate pyrophosphate linker to fluticasone propionate in lysosomal lysate. Stability profile of (B) budesonide ADC and (C) acetal fluticasone ADC in lysosomal lysates for 6 h. Reproduced from [10].

system—Val-Cit-p-aminobenzyl alcohol (PAB) [11]. Designing such type of linker increased the solubility of ADCs to avoid aggregation. In such a system, phosphate-linked budesonide initially released phosphate of budesonide when cleaved with cathepsin B by the consecutive loss of PAB. Compare to this, they developed two different linker. First, a carbonate bridged linker in which budesonide linked to cathepsin B cleavable linker via PAB spacer and another in which budesonide was connected to PAB through an ethylene diamine spacer as shown in Figure 5A. The ethylene diamine spacer, did not undergo intramolecular cyclization to release the budesonide payload because the aliphatic alcohols are less efficient leaving group compared with phenols (Figure 5A).

Moreover, considerable aggregation was observed when the carbonate brigded linker conjugated to -human CD70 antibody, whereas no aggregation observed in the case of Cat-phos (monophosphate and pyrophosphate) diester linkers. This results clearly suggested that the addition of phosphate group to the linker increased the solubility and reduced the possibility of aggregation despite of a lipophilic steroid payload. Furthermore, the Cat-phos diester and carbonate bridged linkers were found stable for 6 h when tested for stability in human plasma. However, the releasing rate of budesonide payload in lysosomal extracts of rats was found superior for both Cat-phos (monophosphate and pyrophosphate) diester linkers (Figure 5B). Initially the monophosphate of budesonide payload

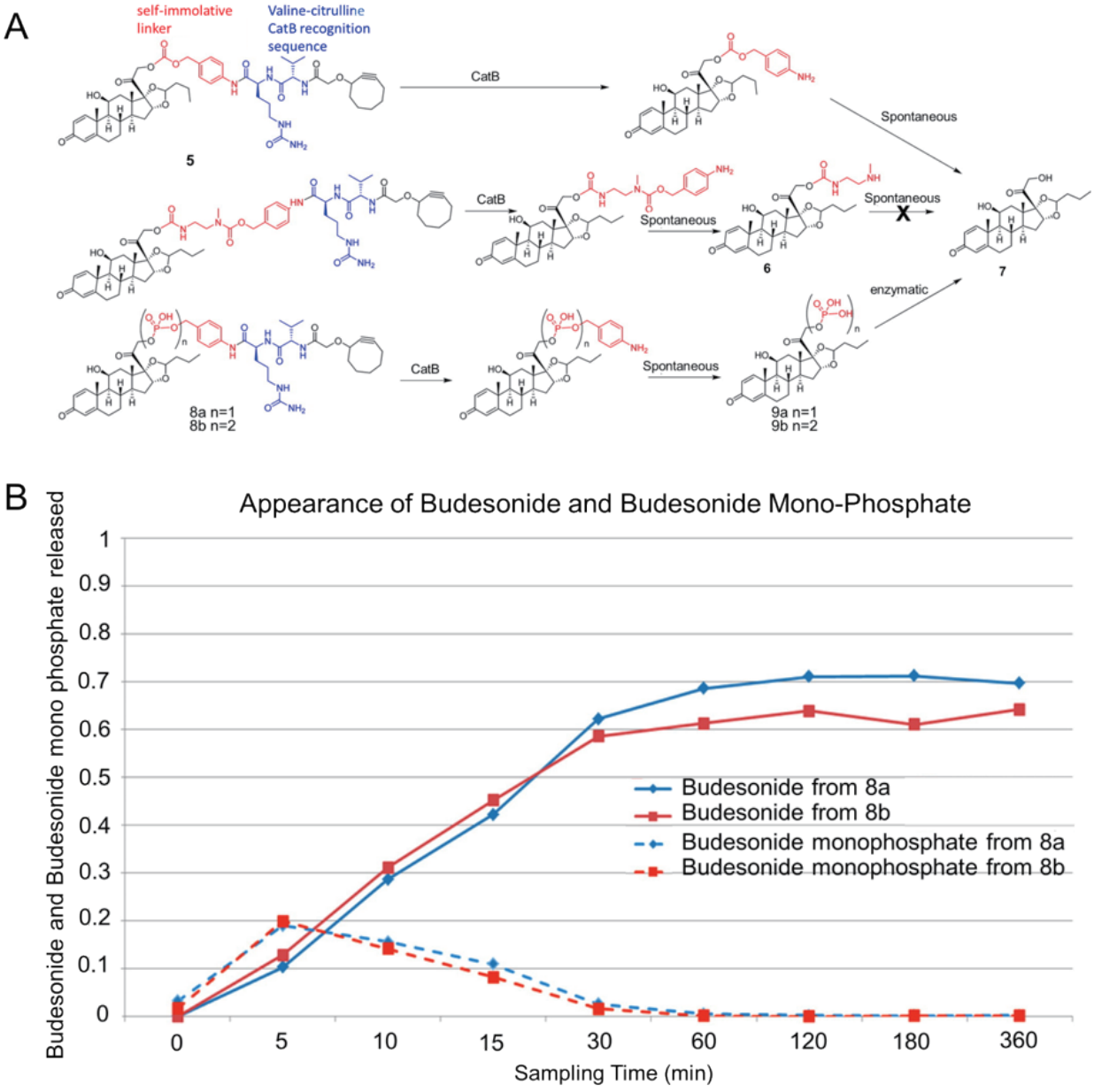

Figure 5. (A) Carbonate bridged, ethylene diamine spacer, and phosphate bridged cleavable linker. (B) Conversion of budesonide Cat-phos linkers to budesonide in rat lysosomal lysate. Reproduced from [11].

was released when cleavage occurred with cathepsin B and monophosphate of budesonide underwent rapid degradation in the presence of phosphatase to deliver free budesonide payload. Therefore, it was an overall dual-enzyme process.

Chimeric molecules can affect the intracellular dilapidation of the targeted protein, but these chimeric moieties are relatively large molecules that may suffer from solubility in an aqueous medium. To increase the aqueous solubility, Dragovich *et al.* [18] developed a new ADC in which a chimeric BRD4 degrader molecule was linked to an antibody through pyrophosphate

diester. This pyrophosphate diester-containing ADC showed high solubility in water and was consecutively hydrolyzed by phosphatase to release the chimeric BRD4 degrader. The ADC showed excellent dilapidation of chimeric BRD4 when it was examined in a PC3-S1 cell line.

Conclusions

In this article, we have reviewed the recently reported cleavable linkers based on two enzymes phosphatase and sulfatase. These linkers are evident to overcome shortcomings associated with traditional linkers such as acid cleavable, reducible disulfide. Although, the development of these linkers in the ADC is limited and not clinically validated, this cleavable linkers associated with ADCs shows high solubility in an aqueous medium due to the anionic nature of the sulfate and phosphate group. Consequently, ADCs offer high stability in human and mouse plasma as well as a reduced possibility of aggregation. Therefore we belive the role of enzymatic sulfatase and phosphatase cleavable linker will play a potential role in the future ADC based diagnosis and therapeutic field.

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Reference

1. Walsh JS, Bargh DJ, Dannheim MF, Hanby RA, Seki H, Counsell AJ, Ou X, Fowler E, Ashman N, Takada Y, Isidro-Llobet A, Parker SJ, Carroll SJ, Spring RD. Site-selective modification strategies in antibody–drug conjugates. *Chem Soc Rev* 2021;50(2):1305–1353.
2. Joubert N, Beck A, Dumontet C, Denevault-Sabourin C. Antibody–Drug Conjugates: The Last Decade. *Pharmaceuticals* 2020;13(9):245–276.
3. Bargh DJ, Isidro-Llobet A, Parker SJ, Spring RD. Cleavable linkers in antibody–drug conjugates. *Chem Soc Rev* 2019;48 (16):4361–4374.
4. Hamann RP, Hinman ML, Hollander I, Beyer FC, Lindh D, Holcomb R, Hallett W, Tsou RH, Upeslacis J, Shochat D, Mountain A, Flowers AD, Bernstein I. *Bioconjugate Chem* 2002;13(1):47–58.
5. Pillow HT, Sadowsky DJ, Zhang D, Yu S-F, Rosario DG, Xu K, He J, Bhakta S, Ohri R, Kozak RK, Ha E, Junutula RJ, Flygare AJ. Decoupling stability and release in disulfide bonds with antibody-small molecule conjugates. *Chem Sci* 2017;8(1):366–370.
6. Wei B, Gunzner-Toste J, Yao H, Wang T, Wang J, Xu Z, Chen J, Wai J, Nonomiya J, Tsai PS, Chuh J, Kozak RK, Liu Y, Yu FS, Lau J, Li G, Phillips DG, Leipold D, Kamath A, Su D, Xu K, Eigenbrot C, Steinbacher S, Ohri R, Raab H, Staben RL, Zhao G, Flygare AJ, Pillow HT, Verma V, Masterson AL, Howard WP, Safina B. Discovery of Peptidomimetic Antibody–Drug Conjugate Linkers with Enhanced Protease Specificity. *J Med Chem* 2018;61(3):989–1000.
7. Jeffrey CS, Andreyka BJ, Bernhardt XS, Kissler MK, Kline T, Lenox SJ, Moser FR, Nguyen TM, Okeley MN, Stone JI, Zhang X, Senter DP. Development and Properties of β-Glucuronide Linkers for Monoclonal Antibody−Drug Conjugates. *Bioconjugate Chem*, 2006;17(3):831–840.
8. Bargh JD, Walsh JS, Isidro-Llobet A, Omarjee S, Carroll SJ, Spring RD. Sulfatase-cleavable linkers for antibody-drug conjugates. *Chem Sci* 2020;11(9):2375–2380.
9. Bargh DJ, Walsh JS, Ashman N, Isidro-Llobet A, Carroll SJ, Spring RD. A dual-enzyme cleavable linker for antibody–drug conjugates. *Chem Commun* 2021;57(28):3457–3460.
10. Kern CJ, Cancilla M, Dooney D, Kwasnjuk K, Zhang R, Beaumont M, Figureueroa I, Hsieh S, Liang L, Tomazela D, Zhang J, Brandish EP, Palmieri A, Stivers P, Cheng M, Feng G, Geda P, Shah S, Beck A, Bresson D, Firdos J, Gately D, Knudsen N, Manibusan A, Schultz GP, Sun Y, Garbaccio MR. Discovery of pyrophosphate diesters as tunable, soluble and bioorthogonal linkers for site-specific antibody-drug conjugates. *J Am Chem Soc* 2016;138(4):1430–1445.
11. Kern CJ, Dooney D, Zhang R, Liang L, Brandish EP,

Cheng M, Feng G, Beck A, Bresson D, Firdos J, Gately D, Knudsen N, Manibusan A, Sun Y, Garbaccio MR. Novel Phosphate Modified Cathepsin B Linkers: Improving Aqueous Solubility and Enhancing Payload Scope of ADCs. *Bioconjugate Chem* 2016;27(9):2081–2088.

12. Corso DA, Pignataro L, Belvisi L, Gennari C. Innovative Linker Strategies for tumor Targeted Drug Conjugates. *Chem Eur J* 2019;25(65):14740–14757.
13. Kolodych S, Michel C, Delacroix S, Koniev O, Ehkirch A, Eberova J, Cianfe´rani S, Renoux B, Krezel W, Poinot P, Muller DC, Papot S, Wagner A. *Eur J Med Chem* 2017; 142:376–382.
14. Burke JP, Senter DP, Meyer WD, Miyamoto BJ, Anderson M, Toki EB, Manikumar G, Wani CM, Kroll JD, Jeffrey CS. Design, Synthesis, and Biological Evaluation of Antibody-Drug Conjugates Comprised of Potent Camptothecin Analogues. *Bioconjugate Chem* 2009;20(6):1242–1250.
15. Hanson RS, Best DM, Wong C-H. Sulfatases: Structure, Mechanism, Biological Activity, Inhibition, and Synthetic Utility. *Angew Chem Int Ed* 2004;43(43):5736–5763.
16. Albin N, Massaad L, Toussaint C, Mathieu CM, Morizet J, Parise O, Gouyette A, Chabot GG. Main Drug-metabolizing Enzyme Systems in Human Breast Tumors and Peritumoral Tissues. *Cancer Res* 1993;53(15):3541–3546.
17. Bodenmiller B, Wanka S, Kraft C, Urban J, Campbell D, Pedrioli GP, Gerrits B, Picotti P, Lam H, Vitek O, Brusniak M-Y, Roschitzki B, Zhang C, Shokat MK, Schlapbach R, Colman-Lerner A, Nolan PG, Nesvizhskii IA, Peter M, Loewith R, Mering WC, Aebersold R, Phosphoproteomic Analysis Reveals Interconnected System-Wide Responses to Perturbations of Kinases and Phosphatases in Yeast. *Sci Signaling* 2010;3(153):rs4.
18. Dragovich SP, Pillow HT, Blake AR, Sadowsky DJ, Adaligil E, Adhikari P, Chen J, Corr N, dela J, Geoffrey C-C, Rosario D, Fullerton A, Hartman JS, Jiang F, Kaufman S, Kleinheinz T, Kozak RK, Liu L, Lu Y, Mulvihill MM, Murray MJ, O'Donohue A, Rowntree KR, Sawyer SW, Staben RL, John W, Jian W, BinQing W, Wei W, Xu Z, Yao H, Yu S-F, Zhang D, Zhang H, Zhang S, Zhao Y, Zhou H, Zhu X. Antibody-Mediated Delivery of Chimeric BRD4 Degraders. Part 2: Improvement of in Vitro Antiproliferation Activity and in Vivo Antitumor Efficacy. *J Med Chem* 2021;64(5):2576–2607.