Exo-Cleavable Linkers: A Paradigm Shift for Enhanced Stability and Therapeutic Efficacy in Antibody-Drug Conjugates

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Abstract

Customized drug delivery systems have become paramount in the rapidly evolving field of precision medicine, and at the forefront of advances in this regard, antibody-drug conjugates (ADCs) present a symbiotic fusion of cytotoxic payloads and monoclonal antibodies (mAbs) facilitated by intricate chemical linkers. The search for ideal linkers that can dexterously provide the dual functionalities of enhancing circulatory stability and facilitating the effective release of the tumor payload is a present and formidable challenge. The valine-citrulline (Val-Cit) linker, which is used in a wide range of ADCs, despite its approval by the Food and Drug Administration, is associated with several inherent drawbacks, including hydrophobicity-induced aggregation, limited payload capacity, and premature payload release. This study presents a paradigm shift from the conventional linear linker archetype by introducing an exo-linker avant-garde approach that repositions the cleavable peptide linker at the exo-position of the PAB moiety. This molecular refinement not only offered the possibility to overcome the intrinsic drawbacks of the Val-Cit platform, but also significantly improved ADC stability, therapeutic efficacy, and pharmacokinetics. *In vitro* and *in vivo* biological evaluations, confirmed that ADCs designed using the exo-linker blueprint significantly attenuated premature payload release, while increasing the drug-to-antibody ratio, even with hydrophobic payloads, and this without inducing pronounced aggregation. Therefore, the fabricated exo-linker represents a significant improvement with respect to traditional Val-Cit ADCs. Moreover, under the influence of enzymes, such as carboxylesterases and human neutrophil elastase, the payload remained stably conjugated to the ADC, underscoring a favorable safety profile and highlighting potential for clinical translatability. Thus, our findings also demonstrate the potential of the novel exo-linker paradigm as well

as the profound implications of nuanced molecular modifications for reshaping ADC design and functionality.

Introduction

Recent advances in targeted therapeutics have highlighted the need for precise drug delivery to maximize therapeutic indices, while reducing systemic toxicity[1]. Central to this avant-garde paradigm is the antibody-drug conjugate (ADC), which is an intricate assembly of monoclonal antibodies (mAbs) and cytotoxic payloads[2]. This hybrid system, fabricated using specialized chemical linkers, represents a major transformation in disease intervention modalities, particularly in oncology and numerous other pathophysiological settings[3]. Further, the transformative prowess of this hybrid system is evidenced by the imprimatur of the U.S. Food and Drug Administration (FDA), which has endorsed a suite of 12 ADC entities for a range of hematological and solid malignancies. Furthermore, over 100 ADC constructs are currently undergoing rigorous clinical evaluation[1-3].

The unique specificity of mAbs for targeting tumor cells is at the forefront of ADC efficacy. Notably, mAbs enhance the potency of ADCs, while expanding their therapeutic window and improving treatment durability; features that clearly superior to those of traditional chemotherapy regimens[1-3]. However, it is important to emphasize that success in such endeavors is inseparable from the selected mAb or payload. Further, the pivot point is the linker, whose architecture modulates a wide range of ADC attributes, from structural homogeneity to pharmacokinetic profiles and therapeutic safety margins, is also an important factor to consider[4]. Therefore, it is important to advance molecular acumen with respect to linker biology in realizing the potential of ADCs. Their bifunctional mandate is clear: they must exhibit molecular stability in the bloodstream and normal healthy tissue while facilitating efficient intercellular payload

63 delivery and showing tumor-specific linker cleavage. This intricate duality has fueled fervent scientific
64 efforts to explore the breadth and depth of linker science beyond the conventional scope of ADCs to
65 encompass a broader spectrum of bioconjugates[5]. Paradoxically, despite the apparent proliferation of
66 linker choices[5, 6], there is a remarkable monotony in clinical adoption. Several FDA-approved ADCs are
67 based on the ubiquitous Val-Cit linker or its derived Val-Ala linkers[7].

68 The mechanism of action of Val-Cit linkers depends on cathepsin B-mediated proteolysis following ADC
69 endocytosis by target tumor cells, and these processes ensure immediate payload release[5]. The stability
70 of this well-established linker has been further confirmed via robust stability assays in primate and human
71 plasma models. However, it is associated with several limitations. The intrinsic hydrophobic predilection
72 of the Val-Cit PAB linker imposes a limit on the allowable payload amount[8]. In particular, common
73 payload linkers, such as Mc-Val-Cit-PAB-MMAE, struggle with modest drug-antibody ratios (DAR = 3–
74 4) and aspirations for higher ratios are thwarted by their hydrophobicity, which leads to aggregation.
75 Additionally, insidious enzymatic interference, which leads to premature linker cleavage and subsequent
76 payload release, further underevaluates Val-Cit chemistry. Notably, a landmark publication by Bristol
77 Myers Squibb highlighted the vulnerability of the Val-Cit linker to carboxylesterase Ces1C, which results
78 in premature payload detachment[9]. Moreover, Zhao et al. revealed an additional issue associated with the
79 aberrant cleavage of the Val-Cit bond involving human neutrophil elastase, and this implies potential ADC-
80 associated off-target toxicity, possibly leading to neutropenia[10]. Several innovative strategies for
81 overcoming the inherent drawbacks associated with the Val-Cit platform have been developed. Most of

these strategies involve the use of hydrophilic polymer scaffolds, such as PEG[11], polysarcosine[12], cyclodextrins[13], peptides[14], and polyacetals[15], which are used to mitigate payload hydrophobicity. Further, pioneering efforts by the Tsuchikama group have led to the establishment of linkers with hydrophilic moieties that exhibit recalcitrance to non-cathepsin B enzymes, with glutamic acid at the forefront[16, 17]. These novel constructs, exemplified by the Ces1C-resistant Glu-Val-Cit[16] and neutrophil elastase-resistant Glu-Gly-Cit[17] platforms herald a new era for linkers. Additionally, groundbreaking link format strategies involving tandem linkers[18], PEG-functionalizing PAB[19], and non-canonical amino acids have also been proposed[6]. However, these strategies have some limitations. For example, the associated synthesis processes are complex and there exists a potential impact of immunogenicity on polymer molecules[20]. Further, with respect to linear tripeptide linkers, the challenge of payload hydrophobicity still exists.

Therefore, in this study, we present a novel linker paradigm that offers the possibility to circumvent the intrinsic drawbacks associated with the Val-Cit linker (Figure 1). Our innovative method involves molecular recalibration, in which a canonical linear peptide-cleavable linker is repositioned at the exo position of the PAB moiety. This groundbreaking exo-linker strategy seems promising for effectively masking payload hydrophobicity by exploiting the intrinsic hydrophilicity of tetrapeptides, including Val-Cit residues. Further, this molecular metamorphosis not only confers Ces1C resistance but also circumvents human neutrophil elastase-mediated premature payload detachment. We highlight the

potential of our novel exo-linker and discuss how molecular finesse can be employed to redefine therapeutic paradigms.

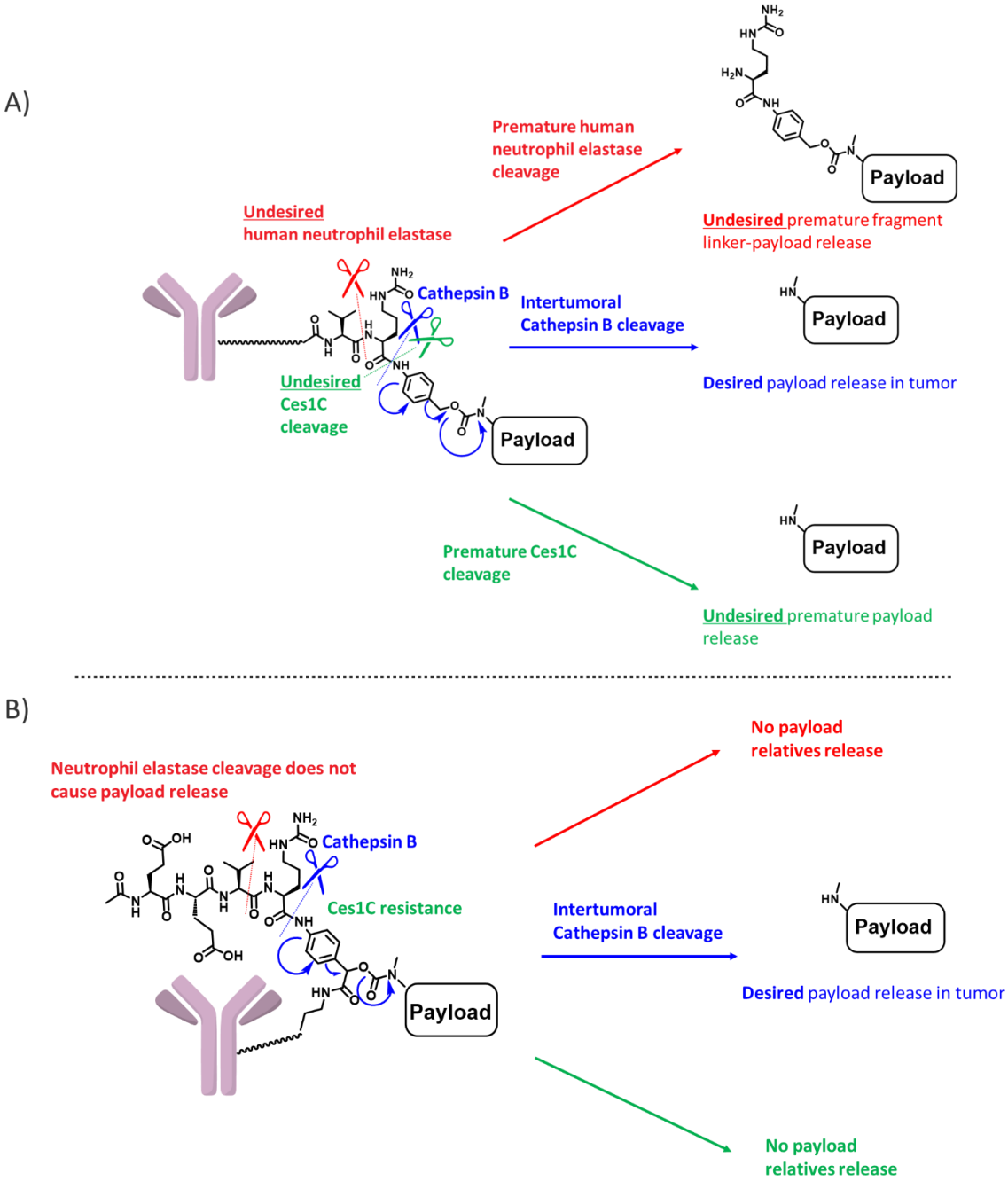

Figure 1. Comparison of Val-Cit PAB and exo-cleavable linkers. (A) Val-Cit PAB linker showing desired cathepsin B cleavage and undesired cleavage mediated by Ces1C and human neutrophil elastase. (B) Exo-cleavable linker showing desired cathepsin B cleavage and undesired cleavage resistance.

Results and Discussion

Evaluation of the physical properties of the novel exo-cleavable linkers

To unravel the potential of the novel exo-linker, we performed a comparative study using a high-DAR (8) ADC alongside the traditional Val-Cit linker. Considering the inherent risk of aggregation associated with the exo-linker owing to its hydrophobic and planar structure, pyrene was chosen as the payload based on its facile synthesis process and amenability to straightforward fluorescence assays. Therefore, by leveraging a known sarcosine molecule[16], we successfully developed a pyrene-based payload and subsequently coupled it to the exo-linker, Exo-EVC-PAB-OH. This was followed by the strategic introduction of a maleimide molecule, which culminated in the synthesis of Mal-Exo-EVC-pyrene. The detailed synthetic routes are described in the Supporting Information (Figures S1 and S2). We also synthesized Mal-Exo-EEVC-pyrene. For validation, control molecules, namely Mc-VC-PAB-pyrene and linear Mc-EVC-PAB-pyrene, were also prepared. ClogP and AlogP evaluations using a previously reported procedure[21] highlighted the distinct hydrophilicity of the exo-linker pyrenes. Using these payloads, ADCs with a DAR of 8 were synthesized via interchain-break conjugation. The fundamental work of Lyon et al. highlighted the existence of a correlation between ADC retention time during hydrophobic interaction chromatography (HIC) and systemic clearance, suggesting that accelerated retention times during HIC HPLC are indicative of favorable hydrophilic properties[22]. Notably, the significantly faster retention dynamics of trastuzumab-exo-EVC-pyrene (ADC (1)) and trastuzumab-exo-EEVC-pyrene (ADC (2))

relative to those of trastuzumab-VC-pyrene (ADC (3)) and trastuzumab-EVC-pyrene (ADC (4)), highlighted their hydrophilic attributes. Further, analytical findings based on size-exclusion chromatography (SEC)[23] revealed a strong disparity in aggregation, with ADC (3) and ADC (4) exhibiting pronounced aggregation, while aggregation profiles of ADC (1) and ADC (2) showed no alterations relative compared to that of native trastuzumab. In a Ces1C-enriched mouse plasma environment, the exo-linker exhibited commendable stability. The variation of the concentration of free pyrene-related compounds remained below 5% even after 4 d of incubation. This robustness was complemented by the retention of cathepsin cleavage, as described in the Supporting Information (Figure S3). Notably, even though ADC (1) and ADC (4) have nearly identical chemical formulas, subtle repositioning of the cleavable linker yielded dramatically different results. This is consistent with our preliminary hypothesis that the masking effect inherent in the cleavable peptide site, in synergy with the structural intimacy between the antibody and payload, collectively enhances ADC properties.

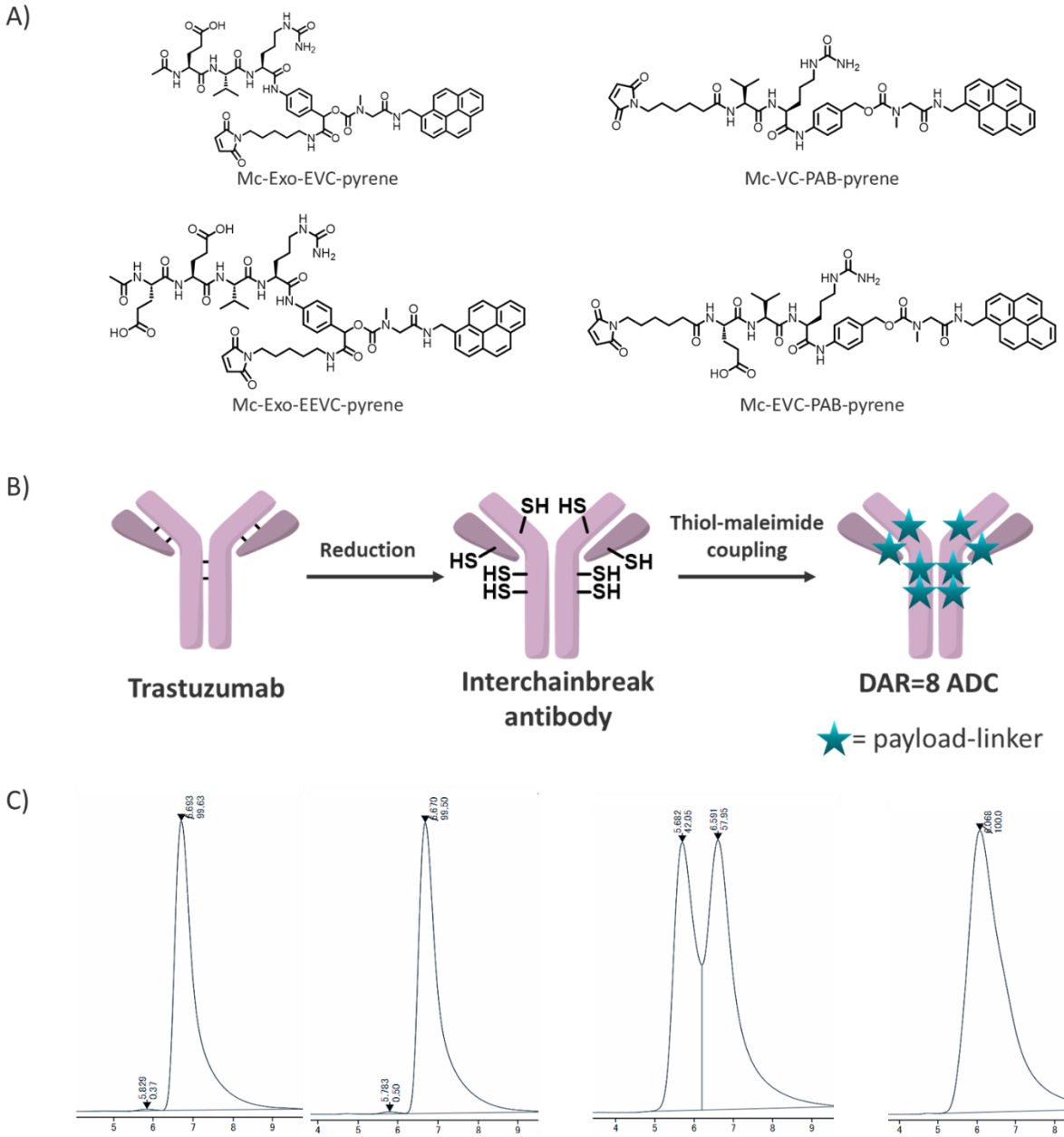

Figure 2. Comparison of the physical properties of Val-Cit PAB and exo-cleavable linkers. (A) Payload-linker structures. (B) Illustration of the synthesis of ADC with a DAR of 8. (C) SEC analysis of ADCs, Exo-EVC-pyrene ADC (left), Exo-EEVC-pyrene ADC (second left), EVC-pyrene ADC (second right), and VC-pyrene ADC (right).

Table 1. Summary of the comparison of the physical and mouse plasma stabilities of Val-Cit PAB and exo-cleavable linkers.

Antibody Conjugates	Linker-payload	ClogP of linker-payload	AlogP of linker-payload	HIC retention time of ADC	DAR in HIC	Aggregation in SEC	Released payload in mouse plasma
Trastuzumab	-	-	-	5.8 min	-	0.5%	-
ADC (1)	Mal-Exo-EVC-pyrene	2.21	2.06	9.7 min	8.0	0.4%	3.5%
ADC (2)	Mal-Exo-EEVC-pyrene	1.06	1.31	9.1 min	7.4	0.5%	2%
ADC (3)	Mc-VC-PAB-pyrene	4.31	3.87	15.0 min	7.9	100%	36%
ADC (4)	Mc-EVC-PAB-pyrene	3.17	3.12	14.9 min	7.8	42%	7%

Application to cytotoxic ADCs

To further validate the potential of the novel exo-linker, we conjugated it with highly cytotoxic payloads, MMAE and exatecan, which are widely recognized and commonly used in commercial ADCs. Given that pyrene evaluation showed encouraging properties, our choice shifted to the exo-EEVC linker. Thus, its use lead to the synthesis of Mal-Exo-EEVC-MMAE (APL-1091) and Mal-Exo-EEVC-Exatecan (APL-1092).

As recently demonstrated, site-specific conjugation tends to provide ADCs with a broader therapeutic window than their random counterparts[24, 25]. Thus, we used the second-generation AJICAP method involving an Fc affinity molecule, to selectively convert native antibodies into site-specific ADCs[26]. Thus, we generated trastuzumab by introducing a thiol group at the Lys248 site. Subsequent conjugation of the Lys248 thiol with APL-1091 and APL-1092 resulted in a targeted DAR of approximately 2 (ADC (5):

156 APL-1091 DAR = 2; ADC (6): APL-1092 DAR = 2). Notably, both APL-1091 and APL-1092 solubilized
157 in the conjugation buffer without the need for the addition of typical co-solvents. This highlighted the
158 universal applicability of the exo-linker, even for antibodies that are potentially unstable in organic
159 solvents. To further demonstrate the beneficial properties of the exo-linker, ADCs with a DAR of 8 were
160 synthesized from both APL-1091 and APL-1092 (Figure S4; ADC (7): APL-1091 DAR = 8; ADC (8):
161 APL-1092 DAR = 8). All these constructs exhibited acceptable HIC retention times and aggregation levels.
162 Even though the known tendency of MMAE to aggregate was evident at a DAR of 8[27], APL-1091 did not
163 exhibit this tendency, confirming its hydrophilic nature.

164

165 ***In vivo* xenograft studies of the novel exo-linker ADCs**

166 In this study, we also rigorously evaluated the fabricated ADCs using NCI-N87 xenograft mice. Thus, we
167 were able to compare the fabricated ADCs with key industry-standard ADCs. For example, APL-1091 was
168 comparable to a trastuzumab ADC (ADC (9)) with a DAR of 4 owing to stochastic interchain disruption,
169 which mirrors the molecular formats of commercial ADCs, such as Adcetris and Polivy[7, 28]. Further, a
170 site-specific ADC (ADC (10)) was designed with Mc-VC-PAB-MMAE as the payload linker using the
171 AJICAP second-generation method in a site-specific manner[26]. This allowed for a clearer comparison of
172 the linkers. Notably, APL-1091-based ADCs showed reasonable antitumor efficacy even at doses as low
173 as 2.5 mg/kg, outperforming their counterparts, such as Mc-VC-PAB-MMAE-based ADCs. Even though
174 the second-generation ADCs synthesized using AJICAP showed comparable tumor inhibition activity

when their payload levels were normalized, the mechanism underlying the superior efficacy of the exo-linker-based ADCs remains to be determined.

In a parallel study, APL-1092-based ADC was evaluated against trastuzumab-deruxtecan (Enhertu), a market-leading ADC with a DAR of 8. The dose was adjusted to match that of the payload. Thus, we observed that the ADC conjugated to APL-1092, ADC (6), showed the most pronounced antitumor activity and demonstrated substantial therapeutic efficacy at a dose of 2.5 mg/kg. Further, it showed a dose-dependent tumor-inhibitory effect on NCI-N87 cells, and when normalized to the incorporated payload amount, it showed superior tumor inhibitory effects compared to trastuzumab-deruxtecan. Furthermore, ADC (8), with a DAR of 8, analogous to trastuzumab-deruxtecan, showed significant tumor growth inhibitory effects at the tested doses, 1.25 and 2.5 mg/kg (Supporting Information, Table S1).

These *in vivo* efficacy studies clearly indicated that the exo-linker enhanced therapeutic efficacy owing to its increased stability in mouse plasma. This efficacy was further enhanced when the exo-linker was combined with second-generation AJICAP technologies.

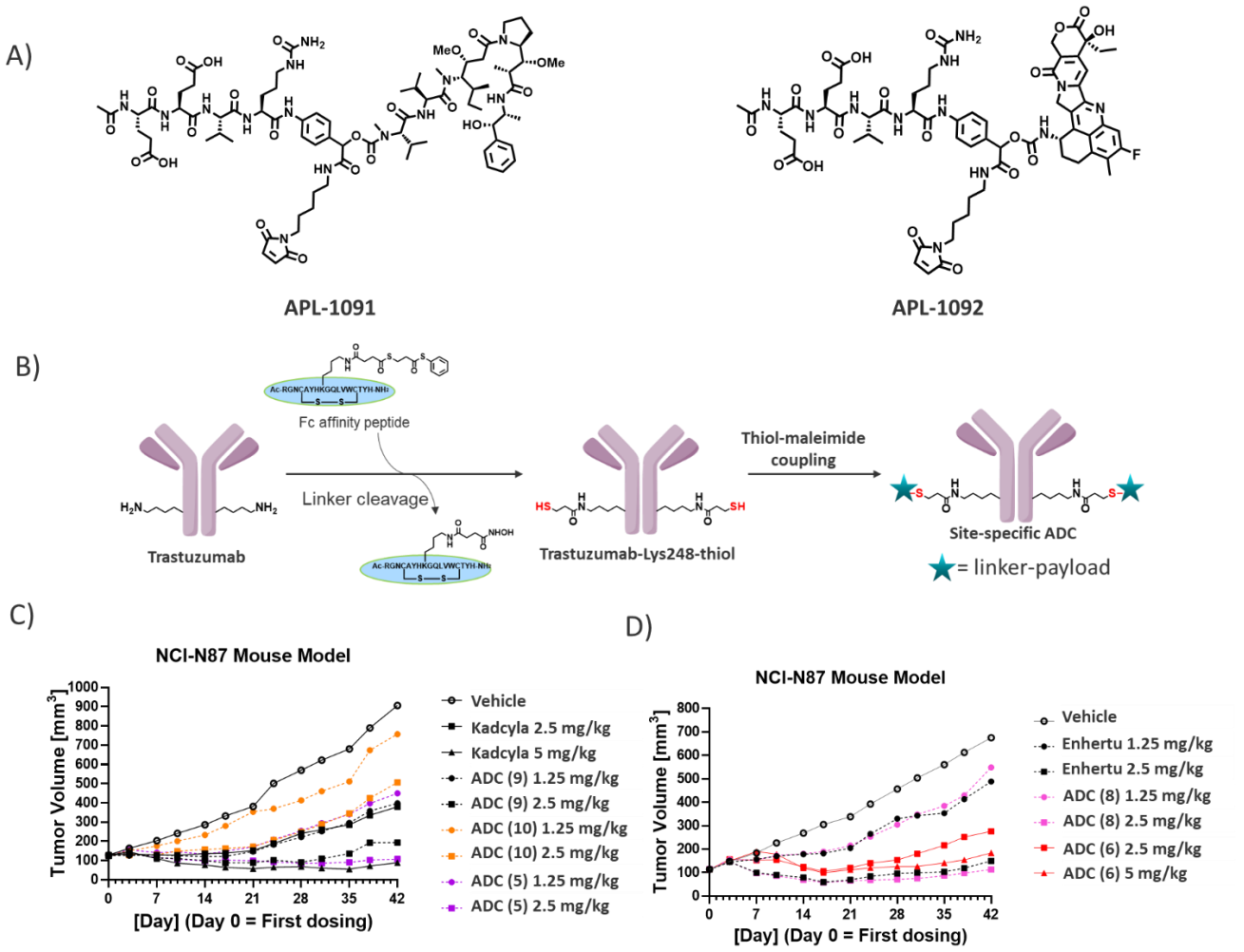

Figure 3. Application to cytotoxic payloads. (A) Chemical structures of APL-1091 (Mal-Exo-EEVC-MMAE) and APL-1092 (Mal-Exo-EEVC-Exatecan). (B) NCI-N87 *in vivo* xenograft studies of MMAE-based ADCs, (c) NCI-N87 *in vivo* xenograft studies of Exatecan-based ADCs.

Table 2. Summary of ADCs.

Antibody Conjugates	Conjugation method	Linker-payload (payload)	ADC retention time in HIC	DAR in HIC	Aggregation in SEC
Trastuzumab	-	-	5.8 min	-	0.4%
ADC (5)	AJICAP	APL-1091 (MMAE)	8.7 min	2.0	1.4%
ADC (6)	AJICAP	APL-1092 (Exatecan)	8.0 min	2.0	1.0%
ADC (7)	Interchainbreak	APL-1091 (MMAE)	11.3 min	7.8	0.5%
ADC (8)	Interchainbreak	APL-1092 (Exatecan)	6.8 min	7.9	1.0%

ADC (9)	Interchainbreak	Mc-VC-PAB-MMAE	11.7 min	4.1	1.2%
ADC (10)	AJICAP	Mc-VC-PAB-MMAE	10.8 min	1.9	1.8%
T-Dxd	Interchainbreak	Deruxtecan	9.1 min	7.8	0.1%

193

194 **Rat pharmacokinetics (PK) studies of the exo-linker ADCs**

195 In the field of rat PK studies, there is need for a methodological shift. Historically, the gold standard
196 methodology for PK studies has been the use of anti-payload antibodies via ELISA. However, the advent
197 of exo-linkers introduces a unique design nuance based on the ability of the linker to mask the payload.
198 However, this feature is associated with concerns regarding steric hindrance, which can prevent efficient
199 recognition by anti-payload antibodies. Therefore, to examine this concern, we performed LC-MS-based
200 ligand-binding assay (LBA).

201 We focused on two AJICAP site-specific ADCs, ADC (5) and ADC (6). Specifically, after dosing, blood
202 samples were carefully collected from the test animals using biotinylated anti-human IgG-Fc fragment-
203 coated beads. This was followed by an accurate LC-Q-TOF MS analysis of the samples to quantify total
204 ADC. The results revealed nuanced interpretations of the DAR transitions derived from the Q-TOF MS
205 deconvolution spectra. Interestingly, for APL-1091-based ADC (5), the DAR remained robustly high at
206 1.9 even on day 21. Meanwhile, APL-1092-based ADC (6) showed a decrease in DAR to 1.6 by Day 21;
207 however, over 80% of its payload remained integrally bound. Notably, having previously delineated the
208 trajectory of total ADC for ADC (10) via conventional ELISA, we sought to bridge the assay

methodologies. Thus, we subjected ADC (10) to LBA (Supporting Information, Figure S30). Although minor variations were observed, with slightly elevated DAR values obtained for ADC (10) via LBA, the divergence was minimal, and the peak DAR difference was only 0.2. Furthermore, a comparative analysis indicated that ADCs (5) and (6), equipped with the avant-garde exo-linker, exhibited better payload linker retention than ADC (10), with the traditional Val-Cit linker.

Conventional total antibody metrics were derived using a well-established anti-human antibody ELISA[25]. The scores for ADC (5) and (6) mirrored the established benchmarks set based on ADC (2) and trastuzumab. Taken together, these findings underscored the transformative stability introduced by the exo-linker in ADC design.

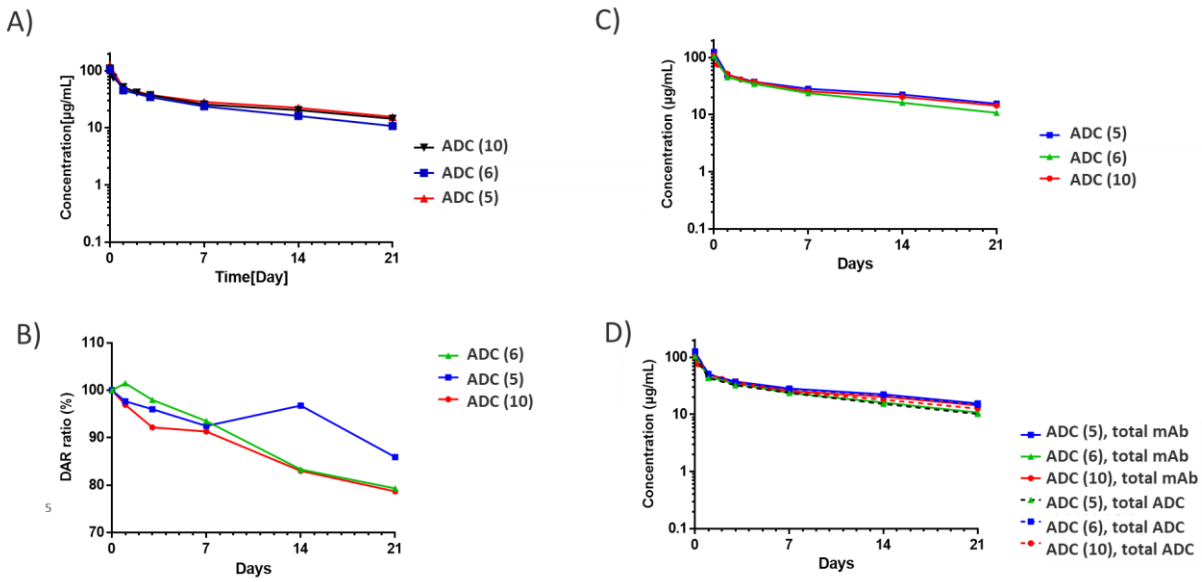

Figure 4. Pharmacokinetic Study of exo-linker-based ADCs in Rats. (A) Analysis of total antibody using ELISA. (B) Assessment of total ADC via LBA assay. (C) Trend in DAR determined based on the LBA/ELISA ratio. (D) Combined trend of total antibody and total ADC.

222

223 **In vitro human neutrophil elastase assay of exo-linker ADCs**

224 In the developing discourse regarding the Val-Cit linker, its undesired cleavage by human neutrophil
225 elastase (NE) has emerged as a pivotal issue[10, 17]. Experimental evidence suggests that NE cleaves the
226 peptide bond nestled between valine and citrulline in the Val-Cit linker. This enzymatic interaction triggers
227 the conversion of the Val-Cit PAB payload to a Cit PAB payload[17, 29], and this supposedly, results in off-
228 target toxicity. In this study, we observed that even in the presence of NE-mediated cleavage, the exo-
229 linker remained attached to the payload linker. This inherent robustness suggested a significant decrease
230 in payload detachment, thereby providing protection against off-target adverse effects. Further, to test this
231 hypothesis, an *in vitro* cytotoxicity assay was designed to detect off-target toxicity caused by NE (Figure
232 5A and B). In this setup, ADCs with Val-Cit linkers and exo-linkers were incubated with NE, while the
233 control ADCs, which were untreated, were incubated with model cells. In HER2-positive model cells
234 (SKBR-3)[30], all the ADCs with Val-Cit linkers or exo-linkers showed cytotoxicity against SKBR-3 cells
235 regardless of NE treatment (Supplementary Figure S31). This indicated that our cell-based cytotoxicity
236 assay was not affected by NE treatment. Next, we performed an assay using the HER2-negative MCF-7
237 cell line. MMAE without linkers was used as a benchmark. In the NE-free environment, the potencies of
238 all the entities, except that of MMAE, remained unchanged (Figure 5C). In contrast, in the NE-treated
239 milieu, AC002 displayed pronounced cytotoxic attributes, exhibiting an IC50 that was an order of
240 magnitude greater than that of MMAE (Figure 5D). It has been postulated that AC002, endowed with Mc-

VC-PAB-MMAE, releases Cit-PAB-MMAE upon exposure to NE. The resulting Cit-PAB–MMAE, potentially driven by the hydrophobic propensity of its Cit-PAB segment, may then exhibit enhanced intracellular penetration compared to MMAE. This trend was also observed for other ADCs with Val-Cit or exo-linkers (Supplementary Figure S31), and highlighted the possibility that NE-induced off-target toxicity may have more pronounced deleterious effects than other premature payload release mechanisms, such as those driven by carboxylesterase.

Fortunately, the findings of this study provide compelling support that the use of the novel exo-linker offers the possibility to overcome these drawbacks. Thus, our study introduces a new era of ADCs with enhanced safety paradigms.

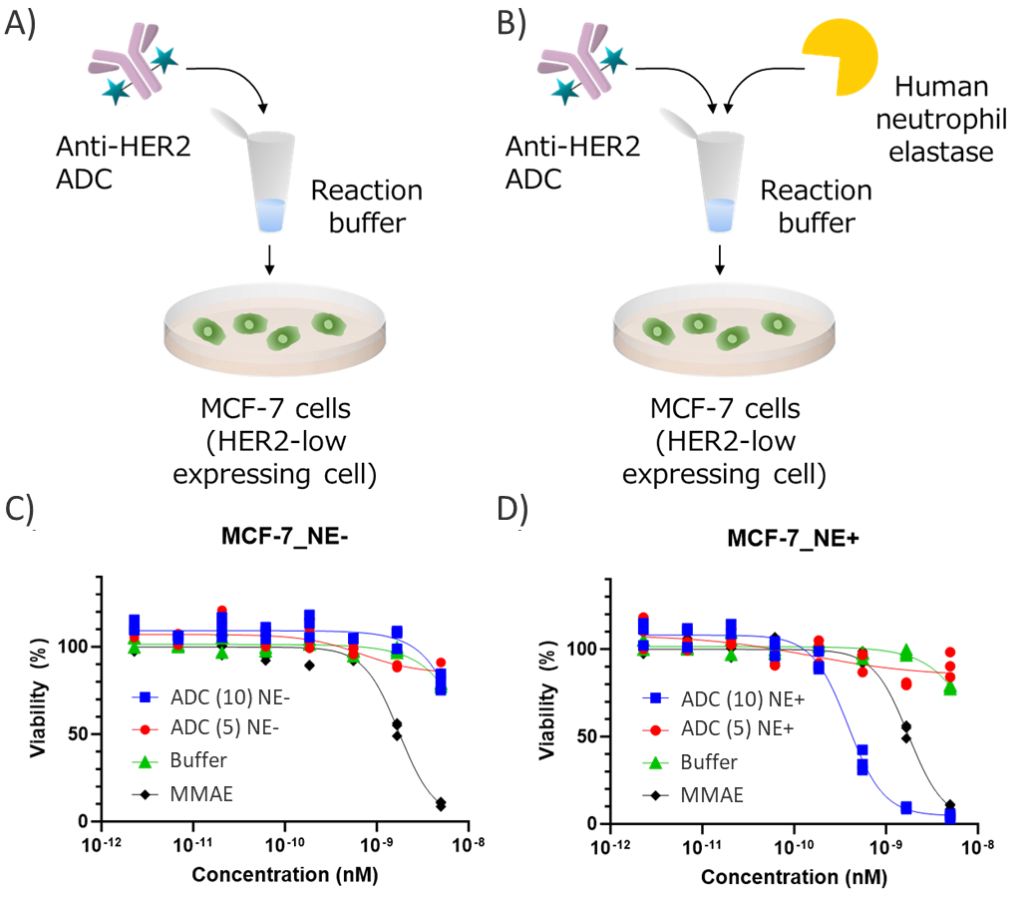

Figure 5. Evaluation of the off-target toxicity of ADCs using an *in vitro* cytotoxicity assay. (A, B) Schematic representation of the assay under (A) NE-depleted and (B) NE-pre-treated conditions. Anti-HER2 ADCs treated or not treated with NE in the NE-reaction buffer and incubated with MCF-7 cells for 6 days. (C, D) Viability of cells incubated with 2.3 pM-5.0 nM of (C) Not treated and (D) NE-treated ADC (10) (blue squares), ADC (5) (red circles), MMAE (black rhombus), and blank buffer (green triangles). Individual values and fitted curves are shown based on the results of triplicate experiments.

The innovative introduction of exo-linkers into ADCs represents a paradigm shift in the field of ADCs as it offers the possibility to address the fundamental issues associated with traditional Val-Cit linkers. Initial comparative studies showed that ADCs synthesized using the exo-linker exhibited superior hydrophilic properties and dramatically reduced aggregation, resulting in improved systemic clearance and robust stability in Ces1C enriched mouse plasma. This superior performance was further confirmed by significant improvements in cathepsin cleavage retention. Further, the conjugation of the exo-linker with established cytotoxic payloads, namely MMAE and exatecan, enhanced its potential as evidenced by the solubility of the constructs, even in the absence of co-solvents. Furthermore, the universal applicability of the exo-linker holds promise for the design of ADCs, including those that are typically hydrophobic. This potential was further validated via *in vivo* xenograft studies, in which the exo-linker ADCs demonstrated enhanced antitumor efficacy, even at reduced doses, outperforming traditional ADCs. In particular, ADCs with APL-1091 and APL-1092 payloads showed the ability to inhibit tumor growth more effectively than leading approved ADCs when normalized for the incorporated payloads. PK studies in rats also provided insights

into the transformative stability conferred by the exo-linker in the ADC design. Thus, this innovative exo-linker design warrants a shift from traditional ELISAs to LC-MS-based LBAs, and demonstrates the superior performance of exo-linked ADCs in terms of payload retention relative to those with the traditional Val-Cit linker. *In vitro* assays using the exo-linker also showed resistance to NE-mediated cleavage, confirming its position as a safer alternative.

In summary, our results underscore the breakthrough potential of exo-linkers in revolutionizing the ADC landscape. Specifically, by offering the possibility to overcome the inherent issues associated with traditional linkers, the exo-linkers present as an avant-garde solution, ushering in a new era of ADCs characterized by improved therapeutic efficacy and safety profiles.

Acknowledgments

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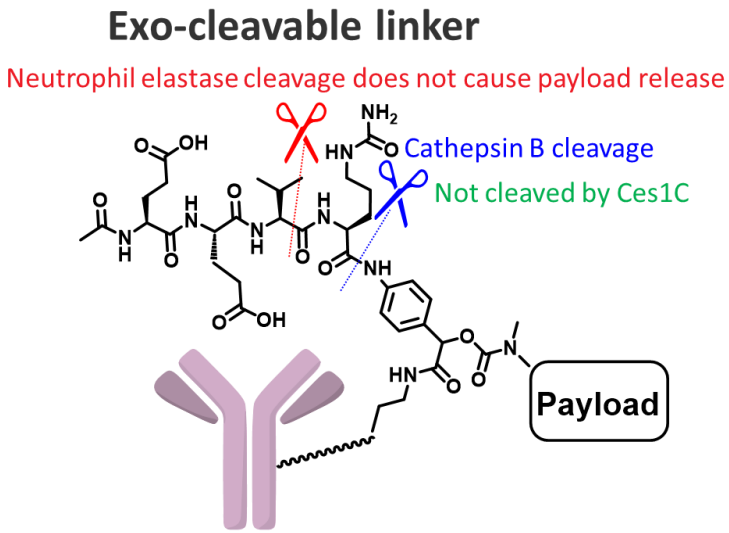
Supporting Information available. Details of the *in vivo* studies, figures supporting exo-linker synthesis, HPLC chromatograms, and QTOF MS analyses.

Conflict of Interest

This work was supported by Ajinomoto Co., Inc.

Table of Contents Graphic



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294 **Synopsis**

295 The authors novel drug delivery approach, refining ADCs for enhanced stability and efficacy. The
296 introduced exo-linker outperforms traditional designs, minimizing payload issues and boosting therapeutic
297 potential, promising improved clinical outcomes.

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