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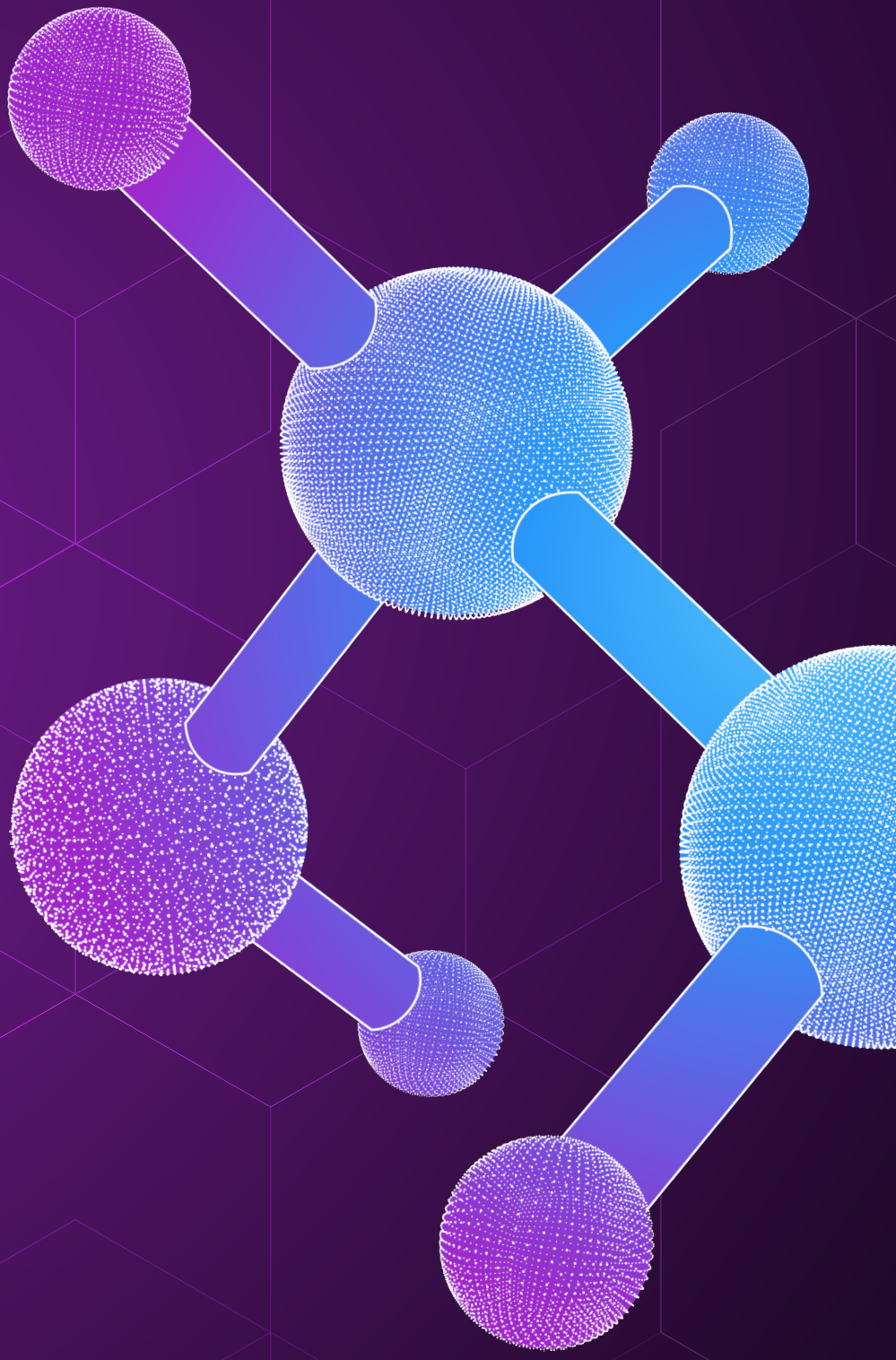

The Brochure Trilogy • Part 2

Advantages of dPEG® in ADC Linker Design

The jinx of DAR, efficacy, clearance, and toxicity.

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Antibody drug conjugates (ADCs) are one of the fastest growing modalities of treatment for various cancers (Han et. al.[1]) as well as non-oncological conditions such as autoimmune disorders (Berkland et al[2]), rheumatoid arthritis and infectious diseases (Chella et al[3].) As the field of precision medicine evolves, the demand for more sophisticated and effective biologics has intensified. This shift necessitates the development of next-generation ADCs capable of overcoming the limitations encountered by their predecessors, such as heterogeneity, poor physicochemical properties, low efficacy, off target toxicity, low target uptake, low systemic stability, high clearance, and low payload capacity. Recent innovations in bioconjugation strategies, payload diversification, and linker technologies have paved the way for a second generation of ADCs with markedly improved performance profiles. Despite these advancements, however, the quest for the ideal ADC design continues.

Linkers, which covalently connect the cytotoxic drug or the effector moiety to the antibody or the targeting moiety, are rapidly gaining recognition as a critical element in an ADC designed for optimal performance (Zhang et. al. [4], Goldmacher et. al.[5]). This has led to numerous innovations in linker design and chemistry over the past decade (Li et. al.[6]). Despite these advancements, however, not all next-generation linkers provide performance improvements. With every new ADC construct, the linker architecture, hydrophilicity and charge characteristics need to be fine-tuned to compensate for the variability imparted by the cytotoxic payload, the payload release mechanism, the antibody used for targeting, and the disease state being targeted.

Of the many types of linkers available – alkyl linkers, sulfonated linkers, polysarcosine (PSAR) based linkers, and polyethylene glycol (PEG) based linkers,

The discrete PEG (dPEG) based linkers have demonstrated performance advantages (dPEG Linker Platform) and are highly customizable.

Vector Laboratories is a leading provider of dPEG linkers and modifiers for ADC development with an array of reactive groups, linker architectures, end capping, ethylene oxide (EO) lengths, and cleavable formats (Diverse portfolio). Selecting the optimal linker can significantly mitigate risks throughout an ADC's development journey.

The advantages of using dPEG linkers to improve linker-payload solubility and conjugation yields, decrease ADC aggregation and hydrophobicity, and increase conjugate stability are showcased in our brochure titled "The curse of low solubility, high aggregation and low stability"

Within the current article, we will present case studies showcasing the benefits of incorporating dPEG into the linker-payload design as an overall strategy to improve efficacy, reduce accelerated clearance, and minimize toxicity. While most examples stem from preclinical studies, we will also examine available results for those conjugates that have advanced to clinical testing, providing a more comprehensive overview of their performance. The data will demonstrate that linker-payload design can provide undeniable advantages in animal models at the discovery stage, and several promising candidates with PEG-based linkers are currently in various phases of clinical trials. While translating pre-clinical results to clinical successes is challenging, these examples highlight the need for continued innovation in linker-payload design and rigorous optimization of each ADC component impacting downstream performance.

Early Experiments that Defined ADC Development

ADCs are designed for the selective delivery of cytotoxic payloads to targeted tumor cells. The greater the number of payloads conjugated to an antibody or the higher the Drug Antibody Ratio (DAR), the more cytotoxins can be delivered to the tumor per binding event resulting in greater efficacy of the ADC. This general concept holds true, but in practice there has been a paradoxical relationship between the DAR and in vivo efficacy.

Early preclinical research in mice with DAR2, DAR4, and DAR8 ADCs prepared by conjugating mc-Val-Cit-PAB-MMAE (vedotin, **Figure 1 top**) to the hinge cysteine residues of anti-CD30 antibodies found that while the in vitro cytotoxicity did indeed scale with DAR, the in vivo efficacy of the DAR8 ADC was inferior to the DAR4 ADC when dosed at the same single (1.0 mg/kg) or multiple (0.5 mg/kg q4dx4) doses. Further studies on the pharmacokinetics of the ADCs found that while the terminal half-lives were not strictly correlated with DAR (16.7, 16.9,

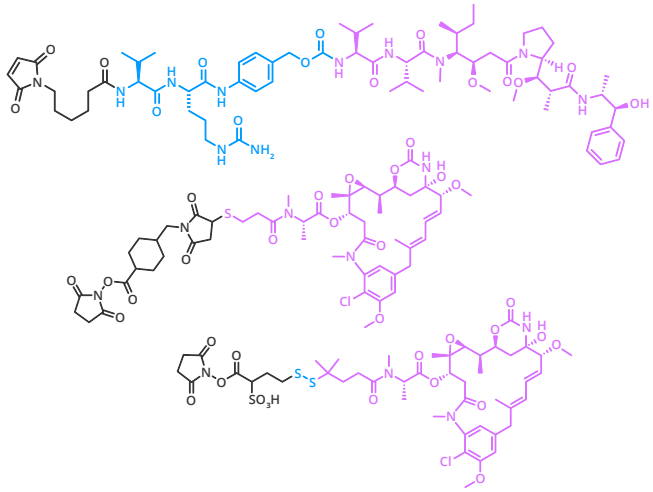

Figure 1: Traditional ADC payloads, vedotin (top), smcc-DM1 (middle), and sulfo-SPDB-DM4 (bottom), resulting conjugates with decreasing exposure and efficacy when conjugated above DAR4.

14.0, and 14.9 days for DAR0, DAR2, DAR4, and DAR 8 species), there was a general trend indicating half-life decreased with increased DAR (11% reduction for the DAR8 species compared to the unconjugated antibody). However, the exposure of the ADCs as determined by the plasma AUC was inversely correlated with DAR and was reduced by 80% for the DAR8 ADC relative to the unconjugated antibody (2638, 2313, 1689, 520 ug-day/mL). It was also found that the mice dosed with the DAR8 ADC at 60 mg/kg exhibited significant weight loss (23%) and distress, while the DAR4 ADC had to be dosed to 120 mg/kg to observe a 17% loss of body weight. Since the DAR4 conjugates were more efficacious than the DAR8 conjugates at the same ADC dose (or ½ the payload dose), this meant the lower DAR conjugate had the greater therapeutic window (Hamblett, et. al.[7])

This observation is not payload specific. This same trend was observed with ADCs prepared by conjugating maytansinoid payloads - smcc-DM1 (**Figure 1**, **middle**) or sulfo-SPDB-DM4 (**Figure 1**, **bottom**), to lysine residues on anti-EGFR antibodies and exploring their activity in mouse models. Once again it was found that the in vitro cytotoxicity scaled with DAR, however dosing DAR3.9 and 6.3 DM1 ADCs in xenograft mice at a single dose of 0.1 mg/kg resulted in 3/6 complete response, while the DAR9.6 DM1 ADC was completely ineffective. PK studies revealed that, similarly to the MMAE payloads, the half-lives were not strictly correlated with DAR (11, 10.3, and 8.7 days for DAR 1.8, DAR 4, and DAR 9.7 species), but there was a discontinuity between "low DAR" and "high DAR" species (a 21% reduction for the DAR8 species relative to the DAR2 species). The exposure was inversely correlated with DAR (13926, 12988, and 1730 ug*h/mL for the DAR1.8, DAR4, and DAR 9.7 species) and there was a disproportionate decrease in the exposure of the high DAR species relative to the low DAR species (an 87% reduction for the DAR9.7 ADC relative to the DAR1.8 ADC). Biodistribution studies revealed that the disproportionate decreases in exposure were due to large increases in clearance by the liver (Sun, et. al.[8]).

Table 1: List of FDA approved ADCs, their reported pharmacokinetic parameters, and commonly observed adverse events in the clinical setting.

ADC	Target	Desease (approved)	Linker	Payload	DAR	T½ (days)	Common AEs (any grade)	Common Grade ≥3 AEs
Zylonia	CD19	R/R DLBCL (2021)	MC-dPEG®-ValAla-PABC(C)	SG3199	2-3	15	Neutropenia, thrombocytopenia, anemia, fatigue, and gamma-glutamyl transferase increase	Thrombocytopenia, neutropenia, anemia, gamma-glutamyl transferase increased, leukopenia, lymphopenia, and hypophosphatemia
Blenrep*	BCMA	R/R MM (2020*)	MC(NC)	MMAF	4	12	Keratopathy, thrombocytopenia, anemia, nausea, pyrexia, blurred vision, increased aspartate aminotransferase	Keratopathy, thrombocytopenia, anemia
Besponsa	CD22	B-ALL (2017)	Ph hydrazone (C)	Ozogamicin	6	12.3	Neutropenia, thrombocytopenia, leukopenia, febrile neutropenia, anemia, and lymphopenia	Neutropenia, thrombocytopenia, leukopenia, febrile neutropenia, anemia and lymphopenia
Enhertu	HER2	HER2+BC (2019)	MG-GlyGly PheGly (C)	Dxd	8	5.7	Nausea, fatigue, alopecia, vomiting, neutropenia, constipation, anemia, decreased appetite, diarrhea, pyrexia, leukopenia, and thrombocytopenia	Neutropenia, anemia, nausea, leukopenia, lymphopenia, and fatigue
Adcetris	CD30	HL (2011)	MC-ValCit-PABC (C)	MMAE	4	4-6	Peripheral sensory neuropathy, nausea, fatigue, neutropenia, diarrhea, pyrexia, vomiting, arthralgia, pruritus, myalgia, peripheral motor neuropathy, and alopecia	Neutropenia, peripheral sensory neuropathy, thrombocytopenia, and anemia
Elahere	FRa	OC (2022)	Sulfo-SPDB (C)	DM4	3.5	5	Nausea, blurred vision, keratopathy, diarrhea, fatigue, peripheral neuropathy, dryeye, and decreased visual acuity	Blurred vision, peripheral neuropathy, and diarrhea
Kadcylia	HER2	MBC (2013)	SMCC (NC)	DM1	3.5	4	Thrombocytopenia, elevated transaminases, fatigue, anemia, and nausea	Thrombocytopenia, increased aspartate aminotransferase levels, and anemia
Tivdak	TF	R/M CC (2021)	MC-ValCit-PABC (C)	MMAE	4	4	Epistaxis, fatigue, nausea, alopecia, conjunctivitis, decreased appetite, constipation, diarrhea, vomiting, peripheral neuropathy, dryeye, and abdominal pain	Fatigue, anemia, abdominal pain, hypokalemia, conjunctivitis, hyponatremia, peripheral neuropathy, and vomiting
Padcev	Nectin4	MUC (2019)	MC-ValCit-PABC (C)	MMAE	3.8	3.4	Fatigue, alopecia, decreased appetite, dysgeusia, nausea, peripheral sensory neuropathy, pruritus, diarrhea, and maculopapular rash	Rash, neutropenia, anemia, and fatigue
Polivy	CD79b	R/R DLBCL (2019)	MC-ValCit-PABC (C)	MMAE	3.5	12	Neutropenia, anemia, and peripheral neuropathy	Neutropenia, anemia, and peripheral neuropathy
Mylotarg	CD33	AML (2003)	Ph hydrazone (C)	Ozogamicin	2-3	2.6	Thrombocytopenia, fatigue, neutropenia, pyrexia, nausea, infection, chills, hemorrhage, vomiting, headache, stomatitis, diarrhea, and abdominal pain	Neutropenia, thrombocytopenia, increased AST/ALT levels, and sepsis
Todelvy	TROP2	TNBC (2020)	SMCC-dPEG$_8$-CL2A (C)	SN38	7.6	0.7	Nausea, diarrhea, neutropenia, fatigue, vomiting, anemia, alopecia, and constipation	Neutropenia, anemia, diarrhea, and leukopenia

These results demonstrate the development paradigm for second-generation ADCs. Conjugating hydrophobic cytotoxins to antibodies imparted unfavorable physicochemical properties, and anything above a DAR4 resulted in non-specific interactions and rapid clearance by non- targeted organs, such as the liver, that not only reduced efficacy but also increased off-target toxicity. These factors increased the required minimum effective dose (MED), decreased the maximum tolerated dose (MTD), and contributed to shrinking therapeutic windows for clinical candidates with DARs greater than four. It is because of this practical limitation on DAR that majority of the second-generation ADCs have DARs M 4 and incorporate highly potent payloads, such as auristatins, maytansinoids, and PBDs, to compensate for delivery deficiencies.

It is important to note that while accelerated clearance and off-target toxicities have been reduced with some second-generation FDA approved ADCs, there is still considerable room for improvement.

Table 1 shows some of the characteristics of the FDA approved ADCs, their reported half-lives, and common adverse events observed in the clinical setting. While there is a range of half-lives, in general ADCs are still cleared much faster than endogenous IgGs. Furthermore, of the 97 ADCs in clinical trials that were terminated since 2000, 84% of them were terminated in phase I or phase II, and the vast majority were discontinued due to lack of efficacy at the MTD or other safety issues (Dumontet et. al.[9]). This highlights the difficulty extrapolating perceived benefits at the discovery and preclinical stages to clinical phase successes and emphasizes the need to de-risk development as much as possible through strategic ADC design, for which dPEG-based linkers have found to be a valuable addition to the ADC toolbox.

The ability to optimize the combination of ADC components is a necessity to develop therapies for different targeted antigens, and cancers. For instance, low DAR ADCs can be useful if higher doses are needed to saturate target mediated drug disposition (TMDD) due to antigen expression in other tissues or if greater tumor penetration is needed. However, the lower DAR can often require more potent payloads, which tend to come with an increase in hydrophobicity, thus linker-payload design is essential to improve stability and physicochemical properties. Conversely, the ability to increase the DAR to deliver more payload may prove useful to increase efficacy if tumor saturating doses can be achieved, for tumors with low antigen expression, for antigens that suffer from poor internalization or inefficient intracellular processing, or for using lower potency payloads with less severe off-target effects.

To achieve higher drug loading, linker-payload design can compensate for, or shield, poor physicochemical attributes to their prevent ADC uptake and clearance by non-targeted tissues. Furthermore, the structures of the cleavable trigger and cytotoxin can be modified to optimize release mechanisms, affinities for intracellular targets, membrane permeability, and P-gp-mediated efflux. However, these structural changes may be incompatible with desirable physicochemical and pharmacokinetic properties of a conjugate without employing strategic linker-payload design. The hydrophilicity, biocompatibility, and modularity of PEG-based linkers have established them as essential tools for mitigating undesirable payload attributes and addressing the challenge of increasing drug-to-antibody ratio (DAR) while maintaining in vivo efficacy.

The following sections will show examples using PEG based linkers as part of a design strategy to construct ADCs with a range of DARs and payloads that maintain in vivo efficacy, exposure, and tolerability. It is notable that two of the approved ADCs, one a DAR2.3 PBD ADC and the other a DAR7.6 TOPO1i ADC, both incorporate PEG8 linkers, and ADC development campaigns are rapidly moving towards even more sophisticated PEG-based architectures as their ability to optimize structure-function relationships becomes more important. The combination of antibody engineering and strategic linker-payload design can yield stable and homogenous ADCs with greater stability and exposure, improved efficacy, and reduced toxicity, and PEG spacers are often a critical component of these designs.

Increasing Efficacy with dPEG-Based Linkers

ADC optimization at the discovery and development phases using strategic linker-payload design relies on the key inter-related attributes of in vivo efficacy, clearance, and toxicity. An ADC's efficacy relies on the interactions of the antibody with its antigen, and the payload with its target. However, in practice there are other factors that influence efficacy such as alignment of DAR with the tumor biology, dosing schedule, tumor heterogeneity, off-site on-target binding and the emergence of drug resistance mechanisms. When evaluating ADCs with different drug loadings, they may be dosed at the same ADC dose or at the same payload dose on a mg/kg basis, and this difference may not be important at low DAR, but it can be substantial at high DAR. Linker-payload design can facilitate the construction of high DAR ADCs that are more efficacious than low DAR ADCs since they can deliver more payload to tumors with low antigen expression, and it can provide low DAR ADCs that are more efficacious than higher DAR ADCs since the total amount of administered antibody can be increased.

Improving the Efficacy of DAR8 Relative to DAR4 – Orthogonal PEG12-Hydroxy Modifiers

Linker-payload design can provide DAR8 ADCs that are more efficacious than DAR4 ADCs. Tubulis has designed linker-payloads with an ethynylphosphonamidate reactive group for stable cysteine conjugation, the Val-Cit-PAB-MMAE trigger-payload, and orthogonal PEG modifiers (**Figure 2**). In this case, rather than the typical terminal methoxy group on the PEG, a terminal hydroxy group was used. This linker-payload was used to prepare conjugates with brentuximab that show enhanced in vivo efficacy and PK when compared to brentuximab vedotin (BV, Adcetris). Both DAR4 and DAR8 ADCs were prepared by conjugating linker-payloads with an orthogonal PEG12-OH modifier to the reduced hinge cysteines of brentuximab, and these were evaluated alongside BV (DAR4) for anti-tumor activity in SCID mice bearing Karpas 299 tumor xenografts. When dosed q3d at 0.5 mg/kg in monkeys.

Figure 2

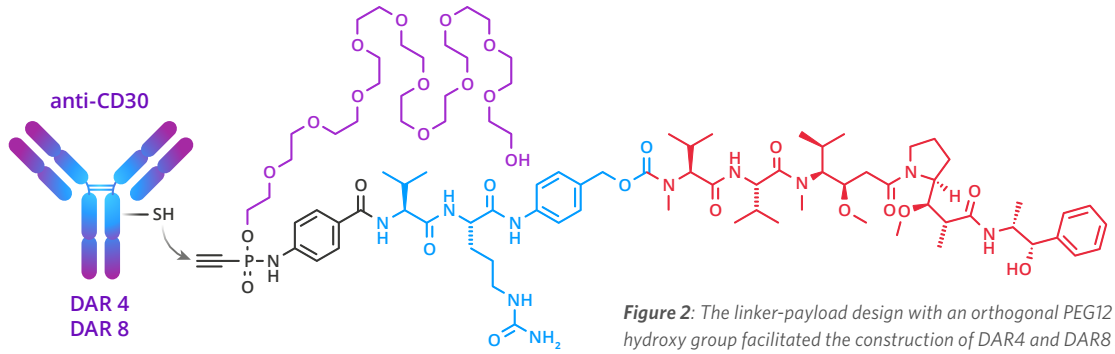

***Figure 2**: The linker-payload design with an orthogonal PEG12 modifier and terminal hydroxy group facilitated the construction of DAR4 and DAR8 ADCs that were more efficacious than BV, with the DAR8 ADC resulting in a 90% remission rate.*

These were then monitored for 74 days. BV induced only temporary and incomplete tumor growth inhibition (TGI) with a 30% remission rate, whereas the DAR4 ADC with designer linker had a higher remission rate of 60%, and the DAR8 ADC with the designer linker exhibited an excellent remission rate of 90% (Ochtrop et. al.[10]). In this case, the ability to prepare a DAR8 ADC with a PK profile similar to the DAR4 ADC increased the amount of payload delivery to the tumor cells and increased efficacy. These results demonstrate the critical role played by the orthogonal PEGs in ADC performance.

Improving the Efficacy of DAR2 Relative to DAR4 – Orthogonal PEG2-Carboxyllic Acid Modifiers

Figure 3

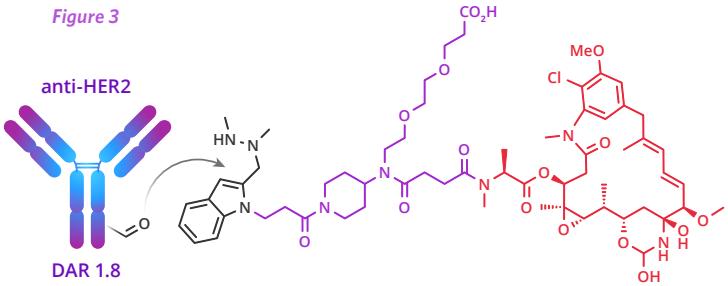

***Figure 3**: Site-specific conjugation and linker-payload design provides a DAR1.8 ADC (CAT-01-106) with greater exposure and better clinical pathology profiles than DR3.8 T-DM1 in preclinical rat and monkey models.*

Additionally, linker-payload design can provide DAR2 ADCs with greater efficacy than DAR4 ADCs. Catalent has applied the SMARTag® technology to develop linker-payloads that provide maytansinoid containing ADCs with better PK and safety profiles than typical DM1-containing ADCs. Linker-payloads were synthesized that contained the HIPS reactive group, a PEG2 modifier in an orthogonal position with a carboxylic acid on the PEG terminus for enhancing solubility, and a maytansinoid cytotoxin (**Figure 3**). This linker-payload (RED-106) was conjugated to an aldehyde tag on the C-terminus of an anti-HER2 antibody to provide a DAR 1.8 ADC (CAT-01-106) and efficacy, PK, and safety profiles were evaluated in various rat and monkey models and compared to DAR 3.8 T-DM1.

In the efficacy and toxicity studies the two ADCs were evaluated at equal payload doses, thus the ADC with the engineered linker-payload was dosed at twice the quantity to compensate for the lower DAR. When evaluated at equal payload doses (3 mg/kg or 6 mg/kg q4w) in mice bearing NCI-N87 CDX , CAT-01-106 was significantly more efficacious, and when evaluated at equal payload doses (single bolus of 7.5 mg/kg or 15 mg/kg) in mice bearing trastuzumab-resistant GA0060 PDX., CAT-01-106 resulted in 100% TGI while both T-DM1 and CAT-01-106 at an equal antibody dose resulted in tumor regrowth. In this particular case, the greater amount of total antibody administered with the low DAR ADC, while providing the same amount of payload, resulted in an increase in efficacy possibly due to greter tumor penetration. In the NCI-N87 CDX model survival rates at 85 days post dose for CAT-01-106 and T-DM1 were 90% and 0%, respectively. In the trastuzumab-resistant GA0060 PDX model survival rates at 110 days post dose for CAT-01-106 and T-DM1 were 90% and 55%, respectively. A comparative PK study in rats administered a single 1 mg/kg dose of either T-DM1 or CAT-01-106 demonstrated the superior half-life and exposure imparted by the linker-payload design and conjugation strategy. The half-life and exposure of the intact ADC for T-DM1 were 4.9 days and 58.3 day*ug/mL, and for CAT-01-106 were 6.6 days and 83.3 day*ug/mL. Thus, the design of CAT-01-106 increased the exposure by 40%. The exposures of intact ADC and total antibody were also evaluated for CAT-01-106 in monkeys given a single dose of 10, 30, or 60 mg/kg, and those of the ADC were only 10-20% less than the total antibody.

To evaluate the safety profiles, rats were given a single IV bolus dose of 60 mg/kg T-DM1 or 120 mg/kg CAT-01-106 and evaluated for loss of body weight and changes in clinical pathology profiles. The rats administered T-DM1 showed clinical signs of morbidly and two of five died while the others lost >20% body weight by day 10 that did not recover by day 12. Conversely, the rats administered CAT-01-106 exhibited fewer clinical observations and recovered their body weight by day 12. The clinical pathology studies also revealed a better safety profile for CAT-01-106, which induced only mild and reversible changes. The ADC also exhibited a similar safety profile when dosed in monkeys at 10, 30, and 60 mg/kg (Barfield et. al.[11]).

Ideally these findings translate to a wider therapeutic window in the clinic, and this is being tested by Triphase Accelerator's TRPH-222, an anti-CD22 antibody conjugated to RED-106, which is in clinical trials for treatment of Relapsed/Refractory Non-Hodgkins Lymphoma Regardless, these results demonstrate that stable site-specific conjugation, a non-cleavable linker, and an orthogonal PEG2-COOH modifier can be combined with a maytansinoid to yield a DAR 1.8 ADC that exhibits greater efficacy and reduced toxicity than the analogous DAR 3.8 ADC (Kadcyla) prepared by the traditional conjugation to lysine residues with the SMCC linker. With promising results. (Hernandez-Ilizaliturri, et. al.[12]).

Improving Efficacy by Combating Drug-Resistance Mechanisms – Linear PEG4 Linkers

The above results show that strategic linker-payload design can be used to find optimal combinations of conjugation site, linker chemistry, and linker properties to produce ADCs with optimal DARs. However, it can also be used to optimize the structure-function relationships of the cytotoxin. Several facets of tumor biology can affect efficacy, and linker-payload design can be used to address these. For instance, heterogenous antigen expression in the tumor mass, can result in incomplete tumor growth inhibition, and many recent development campaigns have focused on proprietary payloads with properties that facilitate diffusion into neighboring cells that do not express the antigen.

Since these modifications are typically associated with an increase in payload hydrophobicity, they can be incompatible with higher DAR and linker-payload design is required to compensate for this.

Another aspect of tumor biology is the potential overexpression of permeability glycoprotein (P-glycoprotein or P-gp), which contributes to the multidrug-resistant (MDR) cancer phenotype and can impact treatment efficacy. In this case the cancer cells overexpress P-gp, either due to inherent or acquired processes, which results in drug efflux and prevents sufficient accumulation of the cytotoxin. In cases such as these, linker-payloads can be devised that are poor substrates for P-gp, and thus more efficacious in MDR models while still exerting their cytotoxic effects.

Figure 4

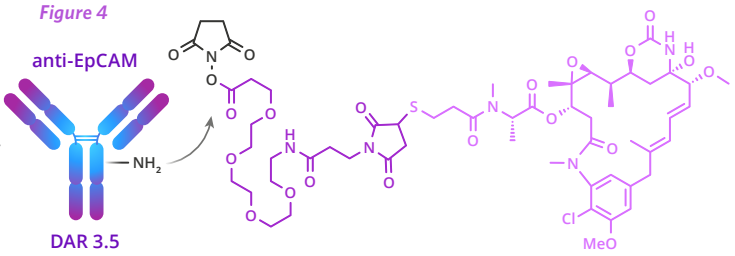

***Figure 4**: Linker-payloads with PEG spacers provide ADCs that are more cytotoxic to antigen-positive cells, less cytotoxic to antigen-negative cells, and more efficacious in multidrug resistant CDX models.*

An example of the latter case was one of the first uses of PEG in the linker-payload design. The activity of the maytansinoid payload accommodates a wide variety of substituents in place of the N-acyl substituent and this has been leveraged to control membrane permeability and drug efflux to modulate the bystander activity and combat the emergence of drug-resistant tumor cells. Incorporating a PEG4 spacer into the linker followed by stochastic conjugation to the lysine residues (**Figure 4**) provided DAR3.5 DM1 ADCs that were more efficacious against multidrug resistant COLO205-MDR tumors in SCID mice when compared to MCC-DM1 (Zhao et. al.[13]).

The above examples show that adequate efficacy is dependent on optimizing ADC properties for the specific tumor biology. Linker-payloads incorporating PEGs can expand the optimization space of ADCs by allowing a greater range of possible DARs and fine-tuning interactions with cellular components, which enhances the likelihood of achieving sufficient efficacy at the maximum tolerated dose (MTD) in clinical trials.

Decreasing Accelerated Clearance and Improving PK with dPEG-Based Linkers

As mentioned in the introduction, accelerated clearance of an ADC can be correlated with reduced potency and increased toxicity, and linker-payload design has proven to be one of the key ADC features driving this process. Clearance of an ADC is typically assessed by monitoring three different species - the intact ADC (the fully conjugated ADC with the initial DAR), the total antibody (total ADC regardless of changes in DAR or conjugated species due to instabilities) and the non-drugged antibody (an antibody that was never conjugated to the linker-payload). Fully conjugated ADCs are cleared from circulation by either conjugate instability or endogenous mechanisms for antibody uptake and proteolysis.

Conjugate instability can result from linker-payload deconjugation, premature payload release, or payload metabolism, and significant differences in the pharmacokinetics of the intact ADC and the total antibody may indicate that one or more of these degradation processes are occurring. Such instability can lead to off-target toxicities, presenting challenges for therapeutic safety and efficacy. The endogenous mechanisms for ADC clearance include receptor-mediated endocytosis (RME) or non-specific pinocytosis. RME initiated by interaction of the Fab domain with its epitope is called target-mediated drug disposition (TMDD) and is the primary mode of action for ADCs. Clearance by this route is highly desired and is the primary driver for ADC efficacy. RME can also be initiated through interaction of the antibody's Fc domain with FcgRs on macrophages, monocytes, and dendritic cells, a major pathway for clearing immune complexes by cells in the organs of the mononuclear phagocyte system (lymph nodes, liver, bone marrow, spleen, lung).

Another highly efficient mechanism for eliminating IgGs is pinocytosis, a nonspecific fluid-phase process performed by endothelial cells.

This process occurs throughout the body, with particularly high activity in highly perfused tissues such as the skin, muscle, and gastrointestinal tract. The last two modes of clearance are generally not desired for ADCs since they result in antigen-independent uptake of the ADC by non-targeted cells and potential off-target toxicities. Significant differences in the pharmacokinetics of the total ADC and the undrugged antibody can suggest drugging the antibody has resulted in an increase in these processes

The undesirable clearance mechanisms reduce the amount of payload

that reaches the tumor and increase the amount of payload that accumulates in healthy tissues, leading to off-target toxicities and subsequently reducing an ADCs therapeutic window. To address these issues, researchers employ advanced antibody engineering, optimized conjugation strategies, and innovative synthetic chemistry techniques. These approaches include modifying the conjugation site, refining conjugation chemistry, adjusting linker length (see Brochure 1), incorporating cleavable triggers, and optimizing the pharmacophore. Strategic design of the linker-payload system, including the incorporation of a PEG component, plays a crucial role in minimizing ADC clearance through undesirable pathways, improving therapeutic performance and safety profiles.

Figure 5

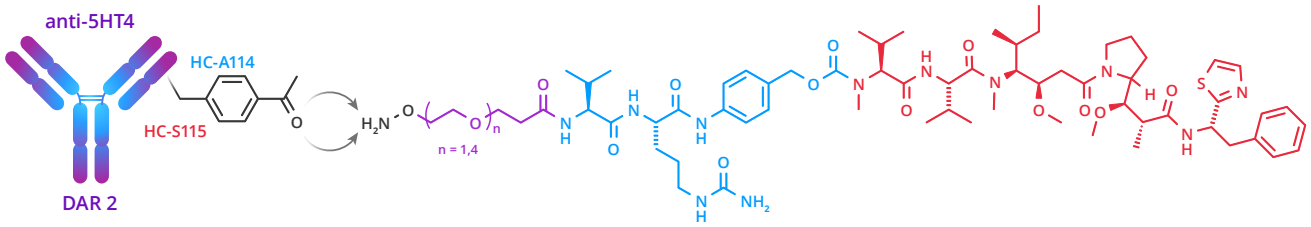

***Figure 5**: Optimal combination of PEG length and conjugation site improve ADC stablilty by preventing premature payload release in rodent.*

Improving Conjugate Stability by Preventing Premature Payload Release – Linear PEG linkers

Conjugate instability is one of the two major mechanisms that clear ADCs from circulation, and linker-payload design has demonstrated benefits in reducing linker-payload deconjugation, premature payload release, and payload metabolism. For example, the length of the PEG spacer was shown to improve conjugate stability with a cleavable Val-Cit dipeptide. Ambrx used genetic code expansion technology to incorporate p-acetyl-phenylalanine (pAcF) into antibodies for site-specific conjugations as part of a strategy for complete ADC optimization. Then, they evaluated linker-payloads with two different PEG lengths conjugated to two different sites on the antibody. Aminooxy-PEG4-Val-Cit-PAB-MMAD or aminooxy-PEG1-Val-Cit-PAB-MMAD were conjugated to two different sites via oxime ligation (**Figure 5**).

Incubation in rodent plasma revealed that conjugate stability depended on both conjugation site and linker length, likely due to premature MMAD release mediated by carboxylesterase 1C. For instance, incubating the ADCs with the linker-payloads conjugated to site S115 in mouse plasma for 72 hours resulted in 25% free MMAD with the PEG4 spacer but less than 5% with the PEG1 spacer. Payload release could be completely abolished by conjugating the linker-payload with the short PEG1 spacer to site A114. This suggests the length of the PEG spacer can be a critical variable for optimizing conjugate stability by reducing premature payload release (Tian et. al.[14]).

Reducing Accelerated Clearance by Reducing Non-Specific Uptake – Orthogonal PEG12-Methoxy Modifiers

As noted in the introduction, several potential antigen-independent mechanisms can contribute to ADC clearance, and for higher DAR species this includes non-specific uptake by the liver. In one important study, Seagen found that the rapid clearance and non-specific hepatic uptake of DAR8 MMAE ADCs was exacerbated by incorporating a linear PEG24 stretcher into the linker-payload. However, this effect was eliminated with orthogonal m-PEG modifiers (Lyon et. al.[15]). A subsequent study found the length of the orthogonal PEG was correlated with improved PK, efficacy, and tolerability, and the PEG12 appeared superior to the PEG4, PEG8, and PEG24, resulting in an ADC with a PK profile that was similar to the undrugged antibody and tolerability similar to the vehicle (Burke, et. al.[16]). Follow up studies were carried out to explore potential mechanisms underlying the rapid clearance and hepatic uptake utilizing an in vitro assay to monitor uptake by Kupfer cells, macrophages in the liver implicated as primary contributors to non-specific clearance of ADCs. A non-binding IgG was reduced and conjugated to mDPR-bAla-glu-PAB-MMAE or mDPR-Lys(PEGn)-glu-PAB-MMAE linker-payloads, generating

Figure 6

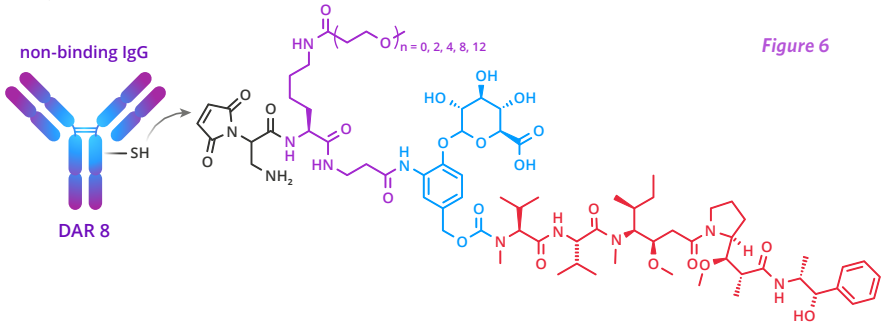

***Figure 6**: The length of the orthogonal PEG modifier in the linker-payload is correlated with improved PK and efficacy and decreased Kupfer cell uptake.*

DAR8 ADCs either without a PEG modifier or with orthogonal PEG modifiers of varying lengths (n = 2, 4, 8, or 12; **Figure 6**). A control IgG was also prepared by capping the reduced hinge cysteines with N-ethylmaleimide. When these ADCs were tested for uptake by rat and human Kupffer cells, uptake was found to inversely correlate with PEG length. ADCs modified with PEG8 or PEG12 showed uptake levels comparable to the unconjugated antibody, whereas those with PEG4, PEG2, or no PEG exhibited a two- to three-fold increase in non-specific

uptake (Meyer et al.[17]). The study also demonstrated that uptake by Kupfer cells was not primarily driven by FcγR binding. An antibody was prepared with well-known mutations that eliminate binding to all FcγR subtypes, and this was conjugated to the mDPR-βAla-glu-PAB-MMAE linker-payload to provide a DAR 8 ADC. The mutations were found to have no effect on Kupfer cell binding or accelerated plasma clearance in rat, thus conjugation induced clearance appears to be independent of FcγR affinity.

This result is distinct from those with some types of tumor-associated macrophages (TAMs), which demonstrated the non-targeted activity of Val-Cit-PAB-MMAE ADCs was reduced by ablating FcγR interactions.

Thus, while the exact role of Fc-mediated non-specific ADC uptake may be different in different tissues, a linker-payload design that incorporates a PEG modifier in an orthogonal position can reduce antigen-independent mechanisms of hepatic uptake, whatever they may be, by the liver's resident macrophages and attenuate the accelerated clearance of an ADC that can result from drugging the antibody.

Reducing Accelerated Clearance by Reducing Non-Specific Uptake – Linear PEG4 Stretchers

The capacity of linker-payload design to minimize antigen-independent uptake by Kupffer cells and enhance clearance is not limited to the use of orthogonal PEG modifiers; linear PEG stretchers can also accomplish this goal with the right combination of cleavable trigger and cytotoxin. Seagen has designed a linker-payload containing a PEG spacer, a novel Val-Lys-Gly cleavable trigger, and a proprietary TOPO1i payload - AMDCPT, designed for bystander killing and activity in MDR models (**Figure 7**, **top**). Linker-payloads with no PEG stretcher, a PEG4 stretcher, or a PEG8 stretcher were conjugated to the hinge cysteines of an anti-CD30 antibody, cAC10. The PEG4 and PEG8 spacers produced DAR8 ADCs with good yields and low aggregation

Figure 7

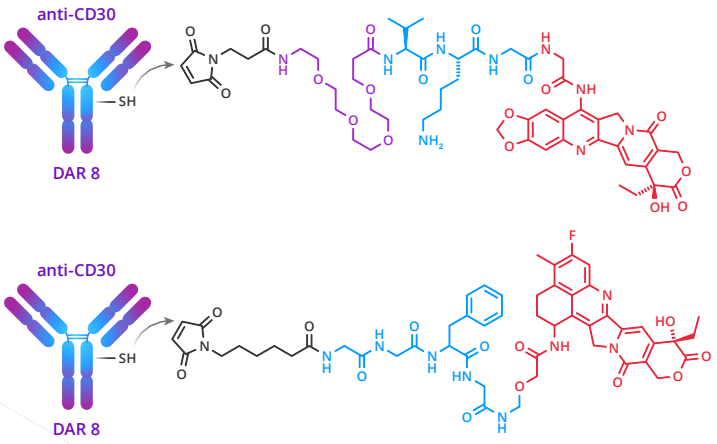

***Figure 7**: The linker-payload in Enhertu (bottom) results in a 70-fold increase in non-specific uptake of the ADC by Kupfer cells, while the linker-payload design on the top results in only a five-fold increase and has minimal impact on ADC PK parameters.*

levels (2–3%), while the linker-payload without PEG caused significant aggregation. Notably, two DAR8 ADCs—one with a PEG4 spacer and another using the mc-GGFG-deruxtecan linker-payload from Enhertu (**Figure 7**, **bottom**)—were tested in a Kupffer cell uptake assay, an in vitro model for non-specific liver uptake. The Enhertu linker-payload caused nearly a 70-fold increase in non-specific uptake compared to the undrugged antibody, whereas the PEG4-Val-Lys-Gly-AMDCPT linker-payload resulted in only a 7-fold increase. Additionally, the pharmacokinetic profiles of a DAR8 ADC with the PEG4-Val-Lys-Gly-AMDCPT linker-payload and an undrugged non-binding IgG were identical in rats given a single 1 mg/kg dose. This indicates that the novel linker-payload, which incorporates a PEG stretcher,has minimal impact on ADC pharmacokinetics, likely due to reduced non-specific liver uptake.

ADCs with both the PEG4 or PEG8 stretcher achieved complete cures in L540cy CDX models after a single 1 mg/kg dose, and both were found to exhibit similar TGI in mice bearing MDR-positive 786-O renal

Figure 8

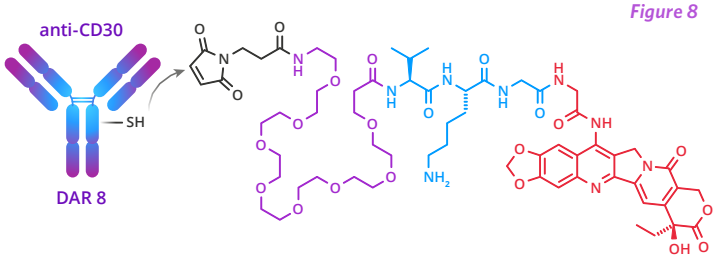

***Figure 8**: A linker-payload design with a PEG spacer, novel cleavable trigger, and proprietary TOPO1 inhibitor provides an ADC with greater activity in MDR models and less hemotoxicity than bretuximab vedotin.*

cell carcinoma tumors when treated with a single dose of 1 and 3 mg/kg. However, ex vivo ADC stability studies in mouse plasma revealed the PEG4 spacer lost 22% of the linker-payload due to deconjugation at 24 hours while the PEG8 spacer lost only 12% of the linker-payload in that timeframe. Thus, the mp-PEG8-Val-Lys-Gly-AMDCPT was chosen for further evaluation (Lyski et. al.[18]).

A comparison of DAR 8 ADCs conjugated to either mp-PEG8-Val-Lys-Gly-AMDCPT (SGN-CD30C) or mc-GGFG-Dxd was conducted using two models: the L428 CDX model with low CD30 expression and the Karpas 299 (CD30-positive)/Karpas BVR (CD30-negative) bystander killing activity model (**Figure 8**). The results demonstrated that the new mp-PEG8-Val-Lys-Gly-AMDCPT linker-payload design was significantly more effective than the linker-payload used in Enhertu. Additionally, the tolerability of non-targeted DAR 8 ADCs was evaluated in rats dosed every 4 weeks at 10, 30, and 60 mg/kg. All doses were well tolerated, with minimal hematological changes observed at the highest dose (60 mg/kg) and negligible weight loss reported (Lyski et. al.[18]).

The DAR 8 SGN-CD30C (**Figure 8**) was also compared to bretuximab vedotin (BV, Adcetris), a DAR 4 ADC based on the same cAC10 antibody with the microtubule-disrupting agent MMAE. Both ADCs demonstrated strong anti-tumor activity in preclinical lymphoma models, and SGN-CD30C induced durable tumor regressions in

DEL-BVR CDX models that are BV-resistant due to the upregulation of MDR1. Repeat dose toxicology studies of the two ADCs were compared in monkeys dosed q1w x 4 with 1 mg/kg BV or 10 mg/kg SGN-CD30C and the hematology parameters indicated the latter was well tolerated at a 10-fold higher dose than the former (Ryan et. al.[19]). While further investigation is needed to determine how these preclinical benefits will translate into clinical outcomes, these findings demonstrate that optimizing the cytotoxin and the cleavable trigger, and incorporating a PEG stretcher into the linker-payload, can yield an ADC with reduced non-specific uptake and without accelerated clearance. In this particuar case, this resulted in ADCs with robust efficacy across various tumor models, favorable tolerability, and promising preclinical profile.

Reducing Clearance by Preventing Deconjugation and Improving Physicochemical Properties – Orthogonal PEG24-Hydroxy Modifiers

The strategy of using orthogonal PEG modifiers to shield undesirable attributes is being applied to other problematic cleavable trigger and payload combinations such as Val-Cit-PAB-Exa. Recall that differences between the PK parameters of the total antibody (regardless of DAR) and the intact ADC can suggest instability, accelerated clearance of high-DAR species with unfavorable physicochemical properties, or a combination of the two. Tubulis has used proprietary conjugation chemistry and orthogonal PEG modifiers to address both potential issues.

The linker technology developed by Tubulis incorporates an ethynylphosphonamidate reactive group for stable cysteine conjugation, a Val-Cit-PAB cleavable trigger, and an orthogonal PEG modifier with a terminal hydroxy group (**Figure 9**). This linker enabled the preparation of DAR 8 ADCs with exatecan as the payload in high yields with minimal aggregation. In vivo stability studies in mice verified the DAR remained stable over seven days unlike Enhertu which exhibited nearly a 50% reduction. The length of the orthogonal

Figure 9

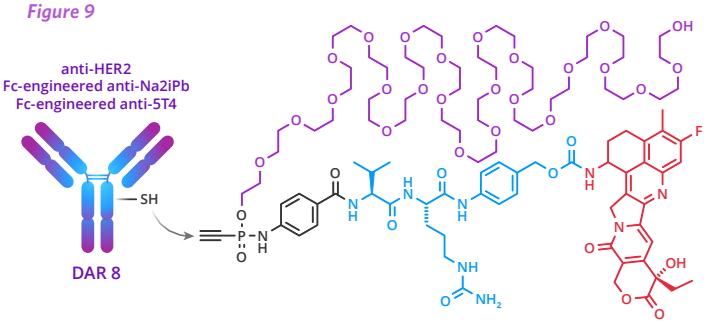

***Figure 9**: Clinical stage ADCs with a linker-payload design that facilitates high drug loading, exceptional stability and PK profiles, and good tolerability in toxicology studies.*

hydroxy PEG modifier was also optimized, and it was found that a PEG24-OH provided greater physical stability than a PEG12-OH, resulting in less aggregation when incubated in buffer. In addition, PK analysis in Sprague Dawley rats revealed it provided greater exposure. The PK studies also revealed the exposure of the total ADC was similar to the undrugged antibody indicating this linker-payload design can facilitate high DAR conjugates by minimizing detrimental effects of drug loading on physicochemical and PK properties. These same preclinical studies revealed an anti-HER2 antibody conjugated to this linker-payload was more efficacious than when conjugated to mc-GGFG-Dxd (Enhertu) at several different doses. When Mice bearing N87 xenografts were given a single dose of 1 mg/kg the PEG24-OH derivative resulted in 8/10 complete responses (CRs) while Enhertu resulted in 0/10 CRs. (Schmitt et. al.[20]).

These linker-payloads have also been conjugated to the hinge cysteines of Fc-silenced antibodies targeting NaPi2b or 5T4 to provide DAR 8 ADCs, TUB-040 and TUB-030, that show long-lasting and durable TGI in a variety of PDX models and were well-tolerated in toxicology studies.

In preclinical studies, the anti-Napi2b antibody conjugated to the linker-payload with the orthogonal PEG24-OH modifier (TUB-040) showed exceptional ex vivo serum stability in rat, monkey, and human.

The ADC also exhibited complete TGI in PDX models of NSCLC and OC at single doses of 1, 3, and 5 mg/kg with no observable changes in body weight, and the tolerability was verified in monkeys dosed q3wx2 with 10 and 20 mg/kg (Helma-Smets et. al.[21]). TUB-040 is currently in clinical trials and TUB-030 is slated to enter clinical trials later this year. These studies demonstrate that extending the length of the orthogonal PEG and the terminal functional group can be optimized to accommodate particularly problematic combinations of cleavable triggers and cytotoxins, and provide DAR8 ADCs with good physiochemical properties, pharmacokinetic profiles, tolerability, and in vivo efficacy.

Reducing Clearance by Improving Physicochemical Properties – Orthogonal Branched PEG12-Methoxy Modifiers

Orthogonal PEG modifiers can help mask undesirable physicochemical properties and improve the pharmacokinetics (PK) of DAR8 ADCs with exatecan and MMAE, and this has also been demonstrated with maytansinoids. In this case, it is shown thatthe impact of the orthogonal PEG modifier depends on the drug-to-antibody ratio (DAR). A recent study evaluated how PEG length and orientation influence the PK of DM1 ADCs at different DARs, comparing them to T-DM1 (Kadcyla, DAR 3.5). Researchers prepared low (DAR 3) and high (DAR 8) ADCs using either NHS-PEG24-maleimide-DM1 or NHS-(PEG12)2-maleimide-DM1 conjugated to trastuzumab lysines, resulting in five ADCs: T-(L24-DM1)3, T-(P(2x12)-DM1)3, T-(L24-DM1)8, T-(P(2x12)-DM1)8, and Kadcyla. (**Figure 10**) In BALB/c mice given a single 10 mg/kg dose, low DAR ADCs (T-(L24-DM1)3, T-(P(2x12)-DM1)3) showed similar half-lives to Kadcyla (9.1, 10.5, and 9.4 days, respectively) but achieved significantly higher exposures (1446, 1356, and 737 d*ug/mL). (Tedeschini et. al.[22]) For high DAR ADCs, PK profiles depended on PEG positioning: T-(L24-DM1)8 had a shorter half-life and lower exposure (6.5 days, 356 dug/mL), while T-(P(2x12)-DM1)8 had a longer half-life and higher exposure (9.2 days, 1051 d*ug/mL) compared to Kadcyla.

These findings suggest that at low DAR incorporating a PEG component can improve PK, but the magnitude may not be substantial, and the positioning of the PEG may be inconsequential. However, at high DAR there can be a substantial impact on PK, and linker-payload designs with long, linear PEG spacers can expose hydrophobic payloads and worsen PK issues, whereas orthogonal PEG placement can shield hydrophobic liabilites and mitigate these challenges. This trend, consistent with Seagen's MMAE studies, points to a potentially universal strategy for improving PK across various payload classes.

Proper ADC exposure at the site of action is critical for efficacy, while premature clearance often hampers performance (Choi et al., Zhang et al.[23]). The ideal clearance rate is context-dependent: rapid clearance, as seen in cases of non-specific uptake, can reduce efficacy and increase toxicity, while slow clearance may indicate poor tumor penetration or TMDD, increasing non-target clearance and toxicity risk. Prolonged circulation also raises the likelihood of healthy tissue exposure to the ADC, leading to potential toxicities from either highly stable ADCs accumulating in non-target tissues or instability issues during extended exposure. Therefore, ADCs should be designed for optimal clearance rather than simply fast or slow clearance, with strategic linker-payload design playing a key role in balancing uptake mechanisms and tissue exposures

Figure 10

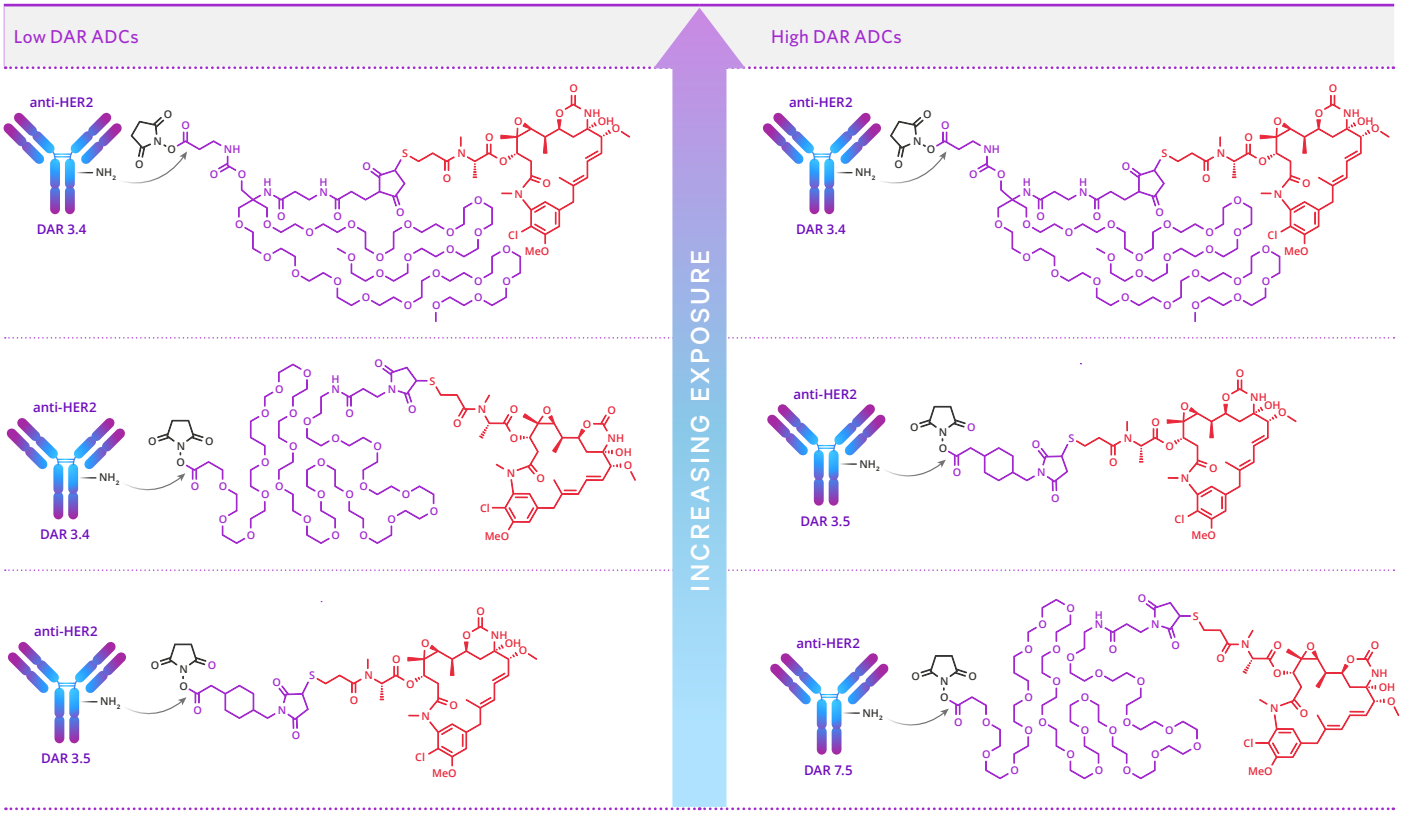

***Figure 10**: Linker-payloads with linear PEG modifiers can exacerbate poor exposure at high DAR while orthogonal modifiers provide good exposure at both low and high DAR.*

Decreasing Toxicity with PEG based linkers:

Toxicity is a critical concern with ADCs and is evaluated during the discovery phase using various methods. Two common approaches are assessing tolerability by loss of body weight and monitoring clinical chemistry measures. Some loss of body weight may be expected, but a loss of 20% in a treated animal is considered a sign of unacceptable toxicity. Additionally, since many dose-limiting toxicities observed in clinical trials involve the hematopoietic compartment or liver, significant and irreversible changes in hematology or serum chemistry of treated animals are cause for concern. These studies often involve a single dose at a higher multiple of the efficacious dose, with dose escalation helping to estimate the therapeutic window.

Figure 12

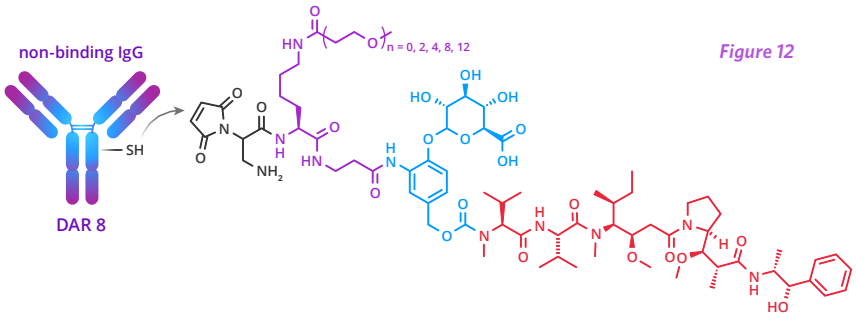

***Figure 12**: The length of the orthogonal PEG modifier in the linker-payload is correlated with increased tolerability, lower MMAE concentrations in non-targeted tissues, and improved clinical pathology markers.*

Toxicity can arise from two major sources:

1. uptake of the ADC by healthy tissues and cells and subsequent endolysosomal processing and payload release, and
2. exposure of healthy tissues and cells to the free cytotoxin.

Uptake of the ADC by healthy tissues and cells can result from expression of the targeted antigen at low levels in those tissues (i.e. tumor associated antigens rather than tumor specific antigens), Fc-mediated processes (as discussed previously), and non-specific pinocytosis (as discussed previously). These sources of toxicity depend on target selection, features of the antibody and ADC that mediate Fc interactions, and physicochemical properties of the ADC. Exposure of healthy tissues and cells to the free cytotoxin can result from conjugate instability in systemic circulation, and diffusion of the free cytotoxin from the tumor after on-site, on-target activity, where it re-enters systemic circulation enroute to the organs responsible for elimination, typically the liver or kidneys.

Figure 11

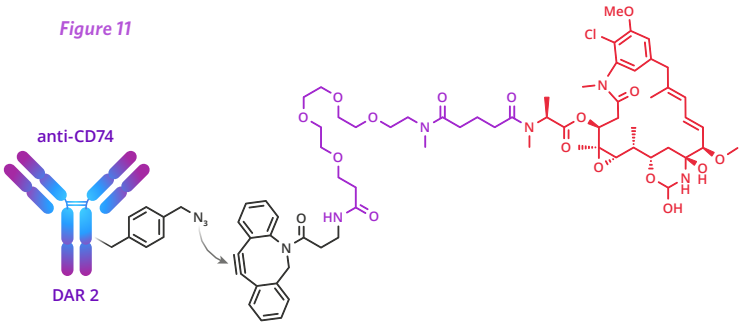

***Figure 11**: Non-cleavable linker-payloads with PEG spacers have good safety profiles and no evidence of off-target tox in preclinical studies.*

These sources of toxicity depend on the properties of the released linker-payload or payload, such as metabolic stability and hydrophobicity. Metabolically stable cytotoxins remain in active form until eventual elimination, while those with metabolic liabilities can be rapidly deactivated. Similarly, hydrophobic payloads diffuse more readily into non-target tissues and persist longer, while hydrophilic payloads remain in circulation and are cleared faster. Incorporating PEG-based components into the linker-payload design can have a substantial effect on many of the aforementioned properties that influence ADC toxicities.

Reducing Off-Target Toxicity by Modifying Physicochemical Properties with Linear PEG4 Stretchers

Non-cleavable linkers ensure that conjugates remain intact during systemic circulation, and the linker-payload that is released after catabolism of the antibody backbone can possess properties that prevent diffusion into antigen-negative cells. Sutro Biopharma combined a PEG4 spacer with a DBCO reactive group for site specific conjugation to engineered pAzMF residues of an anti-CD74 antibody (**Figure 11**). This resulted in a DAR2 ADC with a non-cleavable linker, and the released linker-payload containss a hydrophilic PEG and a zwitterionic amino acid residue from the antibody backbone that render it less cytoxoxic to antigen-negative cells than free maytansine due to reduced membrane permeability. In preclinical studies this ADC had an acceptable safety profile in monkey up to a 10 mg/kg dose and exhibited no evidence of off-target toxicity (Abrahams et. al.[24])

Reducing Off-Target Toxicity by Reducing Free Payload Concentration in Non-Targeted Tissues – Orthogonal PEG-Methoxy Modifiers

As previous studies have shown, an orthogonal PEG modifier can influence the antigen-independent hepatic uptake and accelerated clearance of DAR8 MMAE-containing ADCs, and this can also translate to a reduction in the concentration of the free cytotoxin in non-targeted tissues. A non-binding IgG was conjugated to mDPR-βAla-glu-PAB-MMAE or mDPR-Lys(PEGn)-glu-PAB-MMAE linker-payloads to provide DAR8 ADCs with no PEG modifier or with orthogonal PEG modifiers where n was 4, 8, or 12 (**Figure 12**). These ADCs were then administered to Sprague-Dawley rats to study the effect of the linker on the biodistribution of the free cytotoxin and on the markers of off-target toxicity.

Biodistribution studies assessing the concentration of free MMAE in the liver, spleen, bone marrow, and plasma were conducted and, despite similar overall AUCs, ADCs with longer PEG modifiers had

Figure 13

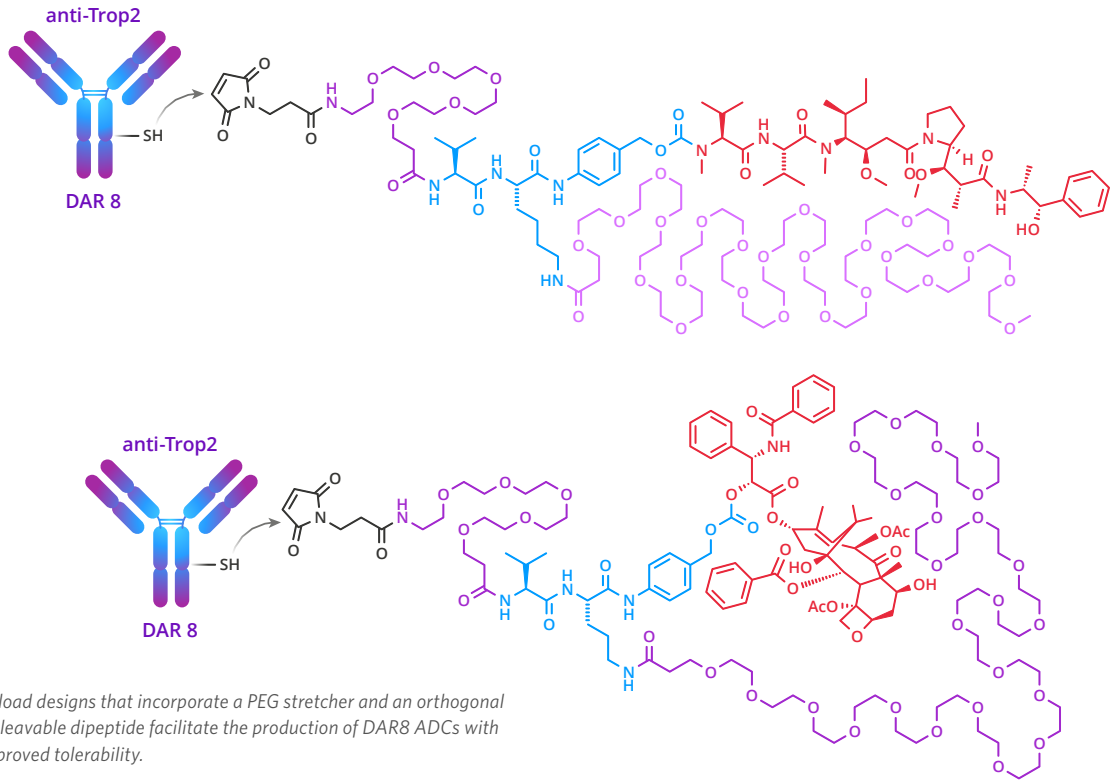

***Figure 13**: Linker-payload designs that incorporate a PEG stretcher and an orthogonal PEG modifier on the cleavable dipeptide facilitate the production of DAR8 ADCs with good efficacy and improved tolerability.*

slower clearance and maintained lower MMAE concentrations in non-targeted tissues and plasma for a longer period of time, while those with no PEG or short PEG modifiers had faster clearance and produced Cmax values that were nearly two-fold higher.

Hematology and serum chemistry results were also correlated with PEG length, and while all ADCs induced neutropenia, the ADCs with the PEG8 and PEG12 spacers had minimal effect on the levels of reticulocytes, platelets, ALT, AST, and ALP. In contrast, rats dosed with ADCs lacking a PEG spacer or containing only a short PEG spacer showed reduced reticulocyte and platelet counts, along with elevated liver enzyme levels. The severity of bone marrow depletion and hepatoxicity were also correlated with PEG length, and the longer PEG modifiers had minimal effects in those areas. The survival rates of rats administered a single 20 mg/kg dose were also correlated with PEG length and ADCs with no PEG or a PEG4 had survival rates of 0% or 15%, while those with PEG8 or PEG12 had survival rates of 100%. In this case, it was proposed that non-specific uptake and catabolism of the ADC by the liver releases the metabolically stable payload, which re-enters circulation, diffuses into non-target tissues, and leads to higher peak concentrations in these tissues, resulting in off-target toxicity. (Simmons, et. al.[25]).

These results demonstrate that both the positioning and the length of hydrophilic PEG modifiers in the linker-payload can be used to reduce non-specific uptake of the ADC by the liver and the exposure of non-targeted tissues such as the liver, bone marrow, and spleen, to the free cytotoxin. These reductions improve preclinical measures of myelosuppression and liver toxicity, and since these are the non-specific effects that tend to be dose-limiting in clinical trials, these types of linker-payload designs could provide a universal strategy to de-risk development of other ADCs

Improving Tolerability by Improving Physicochemical Properties – Linear PEG4 Stretchers and Orthogonal PEG24-Methoxy Modifiers

The MMAE examples above use a hydrophilic glucuronide trigger and orthogonal PEG modifiers to optimize the physicochemical and PK properties of DAR8 ADCs. In contrast, DartsBio integrated the PEG modifier into a cleavable Val-Lys trigger, enabling the use of hydrophobic payloads like MMAE and paclitaxel (PTX), a less potent standalone chemotherapeutic. DartsBio synthesized maleimide-PEG4-Val-Lys(PEG24)-PAB-MMAE and maleimide-PEG4-Val-Lys(PEG24)-PAB-PTX linker-payloads with the PEG24 modifier on the lysine sidechain. These linker-payloads were conjugated to an anti-TROP2 antibody to produce DAR8 ADCs with high yield and monomeric purity (**Figure 13**). Without the PEG modifier, DAR8 conjugates aggregated completely. The MMAE-containing ADC was effective at suppressing tumor growth and when evaluated for tolerability, as assessed by loss of body weight, it was well-tolerated during the standard dosing regimen. Mild weight loss was observed at 60 mg/kg (5%) and was more significant at 80 mg/kg (15%), but these doses are over 10-fold higher than the efficacious dose. The PTX ADC also suppressed tumor growth and showed excellent tolerability, with no weight loss at single doses up to 100 mg/kg, and the MTD was not reached. (Shao et. al.[26]).

This example demonstrates that a linker-payload design that incorporates a PEG stretcher, which spaces the trigger-payload further from the antibody, and an orthogonal PEG modifier on one of the cleavable dipeptide peptide side chains facilitates the production of DAR8 ADCs with hydrophobic trigger-payload combinations and hydrophobic low-potency payloads that exhibit good efficacy and improved tolerability relative to similar DAR8 ADCs.

Figure 14

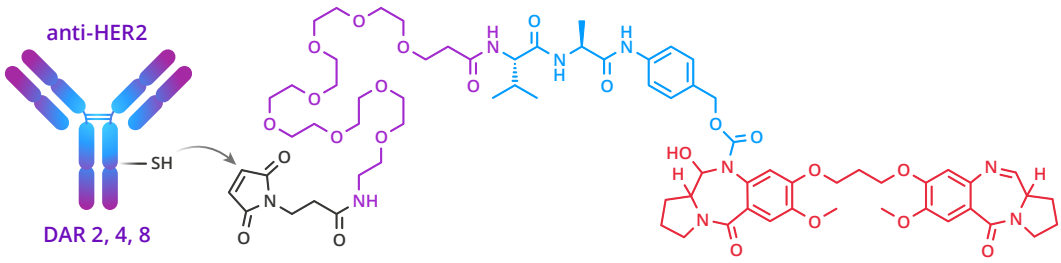

***Figure 14**: The optimal combination of a hydrophilic linker and a less potent but more hydrophobic payload provides ADCs with similar exposures regardless of DAR, efficacies that scale with DAR, and no significant changes in body weight or clinical pathology markers from payload loss.*

Improving Tolerability by Improving Physicochemical Properties – Linear PEG8 Stretchers

In the example above PTX, a lower potency payload used as a stand-alone chemotherapeutic, was used as the payload and this is an emerging strategy for reducing payload-specific toxicity. Payloads that are less potent or belong to classes of cytotoxins already used as standalone therapies, such as deruxtecan (Enhertu) and eribulin (MorAb202), minimize the risks associated with systemic exposure of the free payload, and the toxicities are well known to oncologists and are relatively manageable.

The ability to use less potent payloads has been made possible through advances in linker-payload design that enable higher drug loading to compensate for the low intrinsic potency without detrimental effects on PK.

The PBD dimer SG3650 has low potency and hydrophobicity (clogD = 2.05). The low hydrophobicity and potency of SG3650 make it a good candidate for exploring high-DAR ADCs with low potency PBD payloads, and a linker payload comprised of maleimide-PEG8-Val-Ala-PAB-SG3650 (SG3584) was conjugated to trastuzumab-C239i or the hinge cysteines of trastuzumab to provide DAR 2, DAR 4, and DAR 8 ADCs (**Figure 14**). Indeed, the linker-payload design facilitated high yields and monomeric purities >95% for ADCs with all DARs thus enabling direct comparisons in vivo to determine the effect of drug loading with this linker-payload on tolerability, efficacy, and PK.

The effect of this linker-payload design on tolerability was assessed by dosing the DAR 2 conjugate up to 25 mg/kg in rat and up to 30 mg/kg in monkey, and no significant changes in body weight or clinical pathology markers were observed. The estimated MTDs for ADCs with the SG3584 linker-payload were >25 mg/kg in rats and >30 mg/kg in monkeys. When this is compared to the MTDs estimated for ADCs prepared with SG3400 (10 mg/kg and 4.5 mg/kg) and SG3249 (1.5 mg/kg and 1.0 mg/kg) it lends support to the strategy of leveraging linker payload-designs that accommodate cytotoxins with lower potency to reduce toxicities and improve therapeutic windows (Gregson et. al.[27]).

Pharmacokinetic profiles were also assessed in rats dosed with equal payload amounts, revealing that total antibody clearance and half-life values were consistent across different DARs and only slightly differed from those of the unconjugated antibody. In addition, the PK parameters of the conjugated antibodies were similar regardless of DAR and this suggests that drugging the antibody with this optimized linker-payload design did not have a detrimental impact on PK. When screened in a CDX model at sub-curative equal-payload doses, the three ADCs produced a comparable delay in tumor growth, indicating that increasing the DAR did not adversely affect the ability of the ADC to deliver the cytotoxic drug.

Interestingly, PK analysis revealed that total antibody parameters measured by ELISA (irrespective of DAR) showed greater exposure than conjugated antibody measured by LC-MS/MS, indicating some ADC instability. Released drug was detected in rat serum for DAR 2, DAR 4, and DAR 8 ADCs, though levels were similar across conjugates. Despite this systemic exposure of the free payload, the tolerability studies suggest that the lower potency payload does not present as much of a risk as higher potency payloads if ADC instability is a concern.

A PEG component can be a critical feature of linker-payload optimization to facilitate high drug-loading of low-potency payloads, which can improve tolerability and reduce the risk of insurmountable toxicities in downstream development.

Conclusion

The ultimate goal of ADC design is a clinically successful candidate; however, optimization must begin in the discovery and development phases. Optimizing linker-payload design is a global process where every component—antibody, reactive group, cleavable trigger, payload, spacers, and modifiers—must work seamlessly together to create a fit-for-purpose solution. Each element can be fine-tuned, and often, one component compensates for the limitations of another. While predicting clinical success on the basis of preclinical studies remains elusive, incorporating PEGs into a linker-payload optimization strategy broadens the development possibilities, enhancing efficacy, optimizing pharmacokinetics, and mitigating potential toxicity risks.

Vector Laboratories products for linker-payload design

Vector offers a comprehensive portfolio of dPEG-based precursors and ready-to-use linkers empowering your team with flexible options to meet your ADC development needs. Our intermediate reagents enable the synthesis of bespoke linkers with structures not currently available in our catalog, while our ready-to-use linkers provide solutions for rapid ADC construction. These options support the integration of your proprietary linker-payload combinations and antibodies, streamline linker optimization, reduce resource demands, and accelerate development timelines.

Linear dPEG precursors

Our dPEG precursors and reagents meet our rigorous quality standards and are used for the internal manufacturing of numerous homofunctional and heterofunctional crosslinkers with a variety of dPEG lengths and functionalities. These reagents streamline linker-payload optimization and allow your team to focus efforts on developing proprietary reactive groups, designing linker architectures, and establishing proof-of-concept experiments.

- Amino-dPEG-acids
- Protected amino-dPEG acids
- Protected amino-dPEG-amines
- Amino-dPEG-methoxy
- Acid-dPEG-methoxy
- Amino-dPEG-hydroxy
- Acid-dPEG-hydroxy
- Amino-dPEG-acids

Ready-to-use linear dPEG linkers: This portfolio of ready-to-use linkers allows the rapid construction of ADCs in only two steps,

1. add the payload to the linker
2. conjugate the linker-payload to the antibody

These linkers contain an active ester for reacting with the nitrogen atom of your therapeutic, cleavable trigger, or trigger-therapeutic combination, depending on the starting material your team has in hand. The range of reactive groups for conjugation to the biologic covers the most common conjugation strategies including cysteine conjugation and copper-free click chemistry. These options can utilize either native or engineered proteins, accommodate stochastic or site-specific conjugation strategies, and provide stable conjugates with defined DARs. These ready-to-use linkers allow your team to devote resources to developing proprietary payloads and targeting vectors, and a variety of linkers can be evaluated to rapidly screen for optimal conjugation strategy and linker length.

- Maleimide-dPEG-NHS/TFP
- Bromoacetamide-dPEG-NHS/TFP
- DBCO-dPEG-NHS/TFP
- Azido-dPEG-NHS/TFP
- Protected amino-dPEG-NHS/TFP

Ready-to-use SideWinder linker:

- Maleimide-dPEG4-Glu(TFP)-dPEG24-methoxy

Ready-to-use linear and SideWinder linkers with cleavable triggers:

Vector can also supply the above linkers with the Val-Cit-PABC and Val-Ala-PABC cleavable triggers.

Capabilities with BioDesign

BioDesign is a global conjugate optimization campaign that incorporates dPEG®-based linkers as a critical component to improve synthetic yield, purity, homogeneity, physicochemical properties, pharmacokinetic properties, and pharmacodynamic properties. The success of any new conjugate requires expert knowledge of both chemical biology and linker architecture, and leveraging Vector Laboratories BioDesign™ toolkit gives you access to critical bioconjugation expertise and unique linker IP, alongside ready to use off-the-shelf products. These critical elements help to maximize your development versatility, overcome resource constraints, enable you to manufacture at scale, and decrease your time-to-market.

Vector Laboratories has been making dPEG® products for more than 20 years. We offer a broad range of highly pure dPEG® modifiers, linkers, and spacers.

In addition to our off-the-shelf products, we offer a range of custom products to fit the requirements of your ADC components. Our dPEG products are prepared via a robust and reproducible process and can incorporate a large range of functionalities. Our commercial-scale reactors allow us to manufacture our dPEG products at scales ranging from milligrams (mg) to multi-Kilograms (Kg) with purity suitable for your needs. We are available for customer audits, with appropriate policies and procedures in place to guarantee reproducible results. From initial product development through market release and beyond, we help our customers produce a safe, reliable, and superior product.

References:

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