Bioconjugate Chemistry

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Communication

Tailored Linker Chemistries for the Efficient and Selective Activation of ADCs with KSPi Payloads

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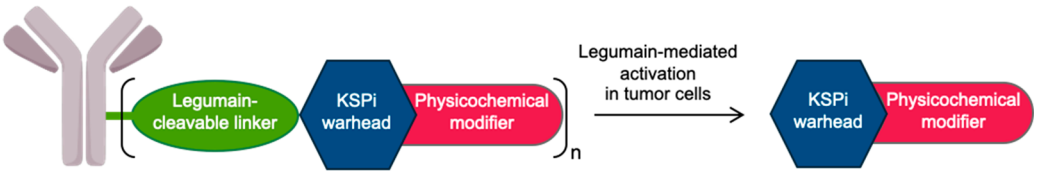

ABSTRACT: Several antibody−drug conjugates (ADCs) have failed to achieve a sufficiently large therapeutic window in patients due to toxicity induced by unspecific payload release in the circulation or ADC uptake into healthy organs. Herein, we describe the successful engineering of ADCs consisting of novel linkers, which are efficiently and selectively cleaved by the tumor-associated protease legumain. ADCs generated via this approach demonstrate high potency and a preferential activation in tumors compared to healthy tissue, thus providing an additional level of safety. A remarkable tolerance of legumain for different linker peptides, including those with just a single asparagine residue, together with a modifier of the physicochemical metabolite profile, proves the broad applicability of this approach for a tailored design of ADCs.

With the approval of, so far, eight antibody−drug conjugates (ADCs), Adcetris in 2011,[1,2] Kadcyla in 2013,[3,4] Besponsa in 2017,[5] reapproval of Mylotarg in 2018,[6,7] Polivy,[8] Padcev,[9] and Enhertu[10] in 2019, and Trodelvy in 2020,[11] plus more than 80 having proceeded to clinical trials, the landscape of ADCs has evolved rapidly during the past decade. However, in parallel to these success stories, unexpected results in the clinical trials have also resulted in a high number of discontinued ADC programs, mainly due to the occurrence of dose limiting toxicities before an efficacious dose was reached. Research in the field has been intensive in order to identify potential strategies to generate efficacious and at the same time well-tolerated ADCs.[12]

To expand the spectrum of therapeutic options, we recently described the utilization of a highly potent pyrrole subclass of kinesin spindle protein inhibitors (KSPis) as a versatile payload class for ADCs with a novel mode of action.[13] However, highly potent and selective ADCs with KSPi payloads could only be obtained with noncleavable linker chemistries so far.[13,14] Currently, ADCs with cleavable linkers are in most cases designed for a proteolytic cleavage by the lysosomal protease cathepsin B.[15] For an efficient drug release, a lipophilic self-immolating spacer unit is required at the cleavage site, resulting in the formation of chinoid entities, which is often associated with aggregation.[14] Furthermore, peptide sequences employed in the linker have shown only moderate specificity for cathepsin B, and premature linker cleavage, e.g., by neutrophil elastase, was observed and has been associated with side effects such as neutropenia in the clinic.[16] Taken together, there is an urgent need for novel cleavable linkers with improved tumor specificity.

Toward this goal, we considered the lysosomal protease legumain as particularly promising. Legumain is an asparaginyl endopeptidase overexpressed in solid tumors, and it has been associated with invasion, metastasis, and poor survival.[17−22] Legumain-cleavable prodrugs have been investigated for extracellular cleavage in tumor stroma; however, drug release may be hampered by the suboptimal pH and the limited stability of legumain under these conditions.[20,23−25] In contrast, legumain-mediated cleavage and activation of KSPi prodrugs released from ADCs has been shown to be highly efficient in the lysosomal compartment of tumor cells with a pH of 4.5−5.5, which is optimal for legumain activity.[26,27] Based on the favorable expression profile of legumain in tumor compared to healthy tissues, its unique cleavage sequence post-asparagine, and its optimal activity at low pH, resulting in intracellular release of the payload, we envisioned that legumain may have a significant potential to enhance the therapeutic window of protease-cleavable ADCs.

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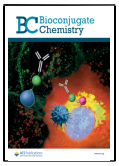

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The goal of the current work was to develop a new generation of protease-cleavable ADCs utilizing legumain-cleavable linkers for specific and highly efficient payload release. Based on the structure−activity relationship (SAR) of small molecule KSP inhibitors the design of legumain-cleavable KSPi ADCs as well as their catabolism to the active metabolite is shown in Figure 1.

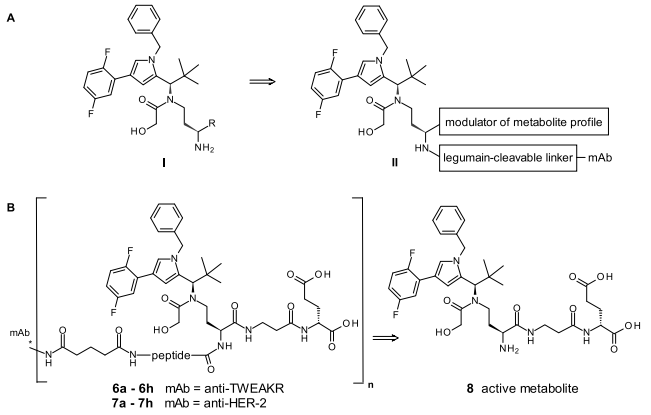

Figure 1. (A) Design of legumain cleavable KSPi ADCs. (B) Lysosomal catabolism of KSPi ADCs providing the active metabolite **8**.

While the free alkyl amino group in the KSPi molecule (structure I in Figure 1A) is crucial for its activity, a variety of substituents are tolerated at position R. The amino group is particularly suitable for the attachment of a legumain-cleavable linker. To provide an active payload metabolite, this amino group has to be released by a specific lysosomal cleavage (Figure 1B). The position R in the structure I has been employed earlier for linkage to antibodies via noncleavable linkers generating highly potent first-generation KSPi ADCs.[13] Here, we used the flexibility of the R position to connect different molecular entities in order to modulate the physicochemical profile of the payload metabolite, e.g., enabling a long retention time within the tumor cells supporting the mode-of-action of KSP inhibitors with low permeability (for details, see Supporting Information).

The synthesis of legumain-cleavable KSPi ADCs from the partially protected KSPi intermediate 1[13] is outlined in Scheme 1A (further details in Supporting Information). Attachment of an orthogonally protected dipeptide, removal of the Teoc protecting group, attachment of legumain-cleavable peptides, and final removal of all protecting groups provided the intermediates **3a−h** (Scheme 1A). These molecules were reacted with 1,1′-[(1,5-dioxopentane-1,5-diyl)bis(oxy)] dipyrrolidine-2,5-dione to yield the activated intermediates **4a−h**, which enabled subsequent attachment to antibodies via its lysine side chains. For proof-of-concept studies, we synthesized the ADCs **7a−h** with an in-house produced version of trastuzumab, the HER2-targeting monoclonal antibody (mAb) employed in Kadcyla.[4] The ADCs **6a−h** were synthesized using ITEM-4,[28] an antagonistic antibody targeting TWEAKR, the receptor of TWEAK [tumor necrosis factor (TNF)-like weak inducer of apoptosis]. TWEAKR is an antigen, which is overexpressed in several solid tumors including non-small cell lung, pancreatic, colorectal, and bladder cancer.[29,30] Further experimental details of the synthesis, analytical characterization, and *in vitro* evaluation are available in Supporting Information. The cytotoxicity of the TWEAKR-targeting ADCs **6a−h** were tested in TWEAKR expressing NCI-H292 lung cancer, BxPC3 pancreatic cancer and LoVo colon cancer cell lines. The HER2-targeting ADCs **7a−h** were tested in the HER2-expressing breast cancer cell line KPL-4 (Scheme 1B). Furthermore, to investigate the specificity of the cleavage of the linker sequences, we synthesized small molecules **5a**, **5b**, **5c**, and **5e** corresponding to the KSPi-linker derivatives of respective ADCs (**6a**, **6b**, **6c**, **6e** and **7a**, **7b**, **7c**, **7e**).

As an initial feasibility study of legumain-cleavable linkers, we synthesized TWEAKR- and HER2-targeting ADCs **6a** and **7a**, respectively, by utilizing a tripeptide linker with a previously described legumain substrate sequence, alanine−alanine−asparagine (Ala-Ala-Asn).[17−25] The N-terminal amino group of this tripeptide is acylated with a dicarboxylic acid, serving as a bridge to the lysine side chains of the antibody. The C-terminal asparagine residue, in turn, is linked to the amino group of the KSPi molecule. Legumain-mediated cleavage of the linker after the C-terminal asparagine is essential for the release of the active KSPi metabolite **8**. Furthermore, the unique cleavage sequence allows for high legumain specificity and discrimination against other proteases. Both the anti-TWEAKR ADC **6a** and the anti-HER2 ADC **7a** demonstrated high potency against the target-expressing cell lines, while the respective isotype control ADCs showed no activity (>300 nM). To stabilize the linker by avoiding nonspecific cleavage at other amide bonds of the linker, we replaced the central alanine residue either by unnatural D-alanine in **6b** and **7b** or an *N*-methyl alanine in **6c** and **7c**. Interestingly, despite these fundamental changes, the ADCs **6b,c** and **7b,c** showed similar potency as compared with the ADCs **6a** and **7a**, respectively, in all cell lines tested. To demonstrate that the cleavage of the highly potent ADC **6c** is mediated by legumain, its epimeric ADC **6d** with unnatural D-asparagine residue at the cleavage site was synthesized. As expected, transformation of the cleavage site led to complete loss of activity in all cell lines tested, demonstrating the essential role of L-configured Asn at the legumain cleavage site for the release of the active metabolite.

The high tolerance for amino acid replacements in position P2 of the tripeptide linker prompted us to completely remove the Ala-Ala dipeptide and attach Asn directly to the antibody via the dicarboxylic acid bridge. Even this major change was tolerated and the ADCs **6e** and **7e** were found to be equipotent with the respective ADCs with tripeptide linkers. To demonstrate that the excellent potency of the ADCs **6e** and **7e** can be attributed to legumain-mediated cleavage, we replaced the Asn residue essential for legumain cleavage with close analogs such as D-Asn (**6f**, **7f**), isosteric Leu (**6g**, **7g**), and homologous Gln (**6h**, **7h**). None of these closely related ADCs showed activity, neither in the TWEAKR nor HER2 series, indicating that the cleavage indeed does not occur in the absence of the Asn residue at the cleavage site. To the best of our knowledge, such linker chemistry as employed in the ADCs **6e** and **7e** has not been described before, making this a pioneering report on a protease-cleavable linker, which requires only a single amino acid residue, Asn, for the legumain-specific cleavage. Next, we investigated the release of the active metabolite **8** from the corresponding small molecule payload-linker prodrugs **5a−5e** in different biochemical assays. In a biochemical assay for legumain-mediated cleavage, **5a** and **5c** were readily cleaved with >50% metabolite formation within

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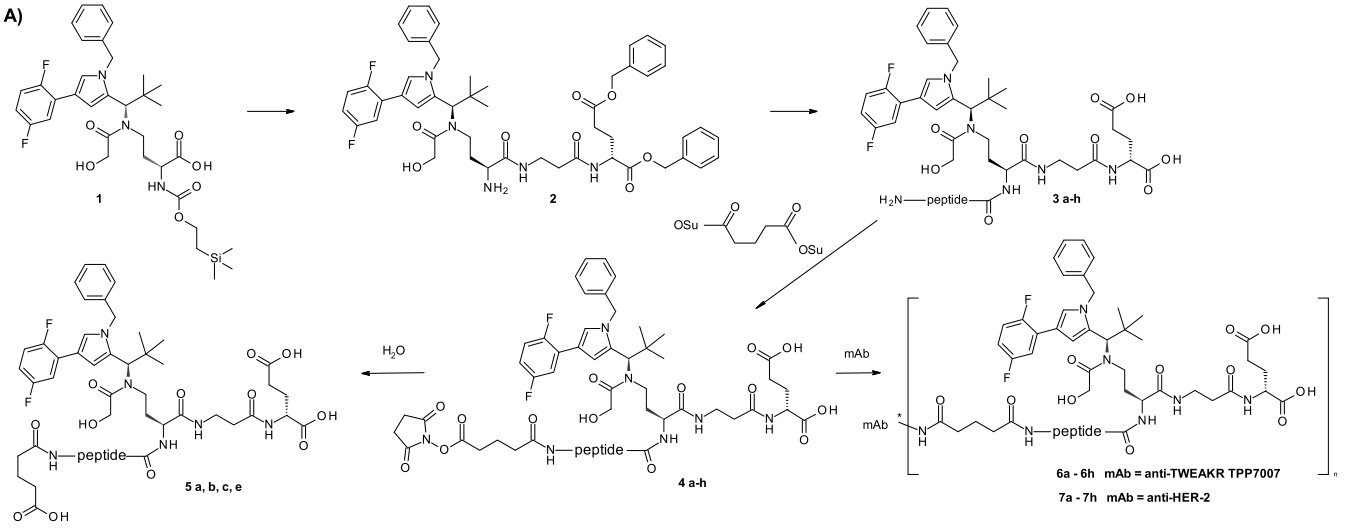
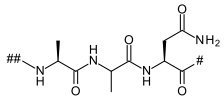
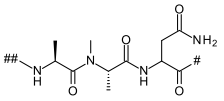
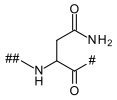
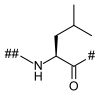
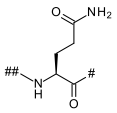
Scheme 1. (A) Outline of the Synthesis of Small Molecule Tool Compounds 5a, 5b, 5c, and 5e and Legumain-Cleavable Anti-TWEAKR ADCs 6a−6h and Anti-HER2 ADCs 7a-7h. (B) Drug-to-Antibody Ratios (DAR) and the Cytotoxic Potency of the ADCs 6a−h in the TWEAKR Expressing Cell Lines NCI-H292, BxPC3 and LoVo and the ADCs 7a−h in the HER2 Expressing Cell Line KPL-4. (C) Concentrations of the Active Metabolite 8 Released from the ADCs 6b* (DAR: 5.7), 6c* (DAR: 5.6), and 6e* (DAR: 7.0) with an Anti-TWEAKR mAb TPP-2658 as Detected in the Cellular Lysate of NCI-H292 Cells and in the Supernatant after 3, 24, 48, and 72 h

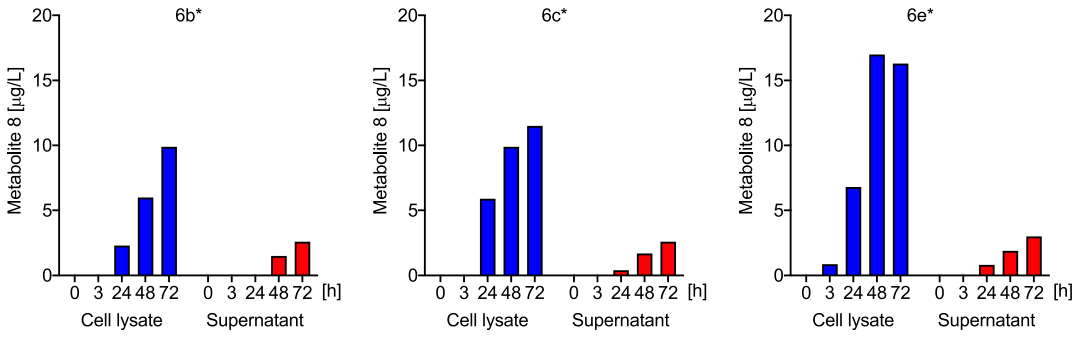
A)



B)

Peptide	SMOL tool compounds 5a, 5b, 5c, 5e	TWEAKR ADCs ADC	DAR	IC_{50} [nM] NCI-H292	BXPC3	LoVo	HER-2 ADCs ADC	DAR	IC_{50} [nM] KPL-4
 L-Ala	**5a**	**6a**	3.2	0.2	0.09	0.04	**7a**	6.3	0.2
D-Ala	**5b**	**6b**	4.6	0.2	0.1	0.03	**7b**	3.0	0.3
 L-Asn	**5c**	**6c**	4.3	0.2	0.09	<0.03	**7c**	5.4	0.3
D-Asn		**6d**	4.5	>300	>300	>300			
 L-Asn	**5e**	**6e**	6.6	0.1	0.07	<0.03	**7e**	6.9	0.2
D-Asn		**6f**	3.4	>300	>300	>300	**7f**	5.4	>300
		**6g**	4.5	>300	>300	>300	**7g**	6.4	>300
		**6h**	4.3	>300	>300	>300	**7h**	6.0	>300

C)



24 h, while **5b** and **5e** were cleaved less efficiently with only ∼7% and 2% metabolite formation under these assay conditions (Table 1). In contrast, upon incubation with neutrophil elastase or cathepsin B, none of the compounds

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Table 1. Release of the Active Metabolite 8 (% of c_{max}) upon *In Vitro* Incubation of Small Molecule Prodrugs with Different Proteases for 24 h

small molecule	legumain %	neutrophil elastase %	cathepsin B %
5a	50.2	0	0
5b	6.7	0	0
5c	68.7	0.7	0
5e	2.2	0	0

5a−**5e** produced a notable amount of the active metabolite **8**, indicating high specificity for legumain-mediated cleavage.

It remains surprising that the ADCs in series b and e, having linkers that appear to be moderately cleaved by legumain, perform equally well with the ADCs of series a and c, where linkers are rapidly and efficiently cleaved by legumain. An explanation could be an exceptionally high legumain activity in the lysosomes of cancer cells, leading to an efficient release of the active metabolite **8** from ADCs even when challenging substrates are employed in the linker. This indeed has been demonstrated by the incubation of NCI-H292 tumor cells with analogous anti-TWEAKR ADCs **6b***, **6c***, and **6e*** comprising the agonistic anti-TWEAKR mAb TPP-2658 (see Supporting Information). As shown in Scheme 1C, all three ADCs released similar levels of the active metabolite **8** in the cellular fraction, which nicely explains the equipotency of the respective ADCs. Notably, this metabolite is released predominantly intracellularly, as indicated by low levels of the metabolite in the supernatant and only at later time points, i.e., after the occurrence of apoptosis. Furthermore, as indicated by the biochemical legumain cleavage assay, the ADC **6e** with a linker consisting solely of the amino acid Asn released high levels of the active metabolite **8**, similarly to the readily cleavable ADC **6c** (Figure 2A,B). In contrast, both ADCs were relatively stable upon incubation with cathepsin B and neutrophil elastase. The high specificity of legumain-mediated cleavage of the ADCs **6a**−**c**, **6e**, **7a**−**c**, and **7e** and particularly the lack of cleavability by neutrophil elastase, which has been associated with neutropenia in cathepsin B cleavable ADCs,[16] may lead to a better safety profile of legumain-cleavable ADCs.

Since liver toxicity is another major side effect of ADCs, we exemplarily tested the cleavage and metabolite formation in a lysosomal preparation obtained from rat liver cells. As a positive control, we used a previously described cathepsin cleavable ADC (ADC1.3 in ref 13), which showed ∼85% release of the active metabolite **8** in our assay. In contrast, the legumain-cleavable ADCs **6a**−**c** and **6e** were found to be relatively stable; the ADC **6a** released ∼18% of the active metabolite **8** after 48 h, whereas the other ADCs with modified linker sequences showed even higher stability. The ADC **6c** released only ∼5% of the active metabolite **8**, and in the case of the ADCs **6b** and **6e**, less than 1% of the active metabolite was released (Figure 2A,B).

The high potency of ADCs associated with efficient metabolite formation in tumor cells together with low levels of the active metabolite in the liver, indicated a preferential activation of the ADCs in tumor tissue compared to healthy organs and thus potentially a higher therapeutic window. Inspired by these results, we next investigated the therapeutic potential of the legumain-cleavable anti-TWEAKR ADCs *in vivo*. Toward this goal, we compared the ADCs **6b**, **6c**, and **6e** with different substrate sequences side-by-side in the NCI-H292 NSCLC and Ku-19-19 urothelial cancer xenograft models in mice.

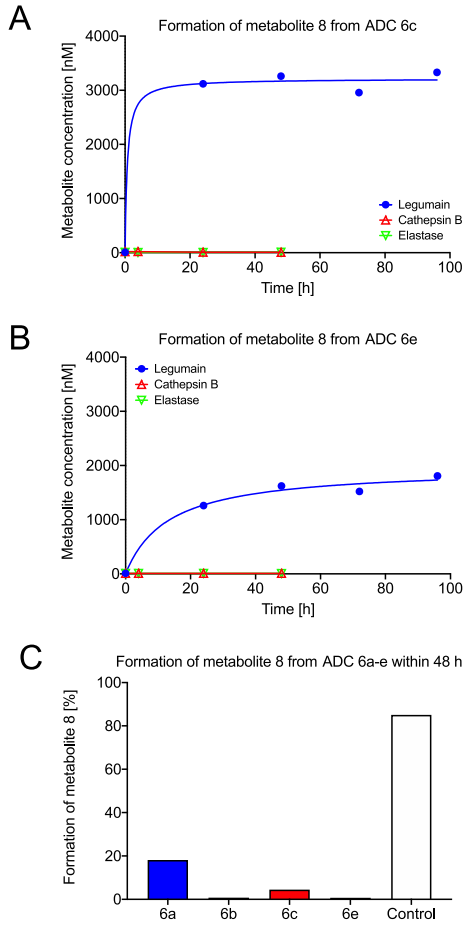

Figure 2. (A,B) Release of the active metabolite **8** from the ADCs **6c** (A) and **6e** (B) upon incubation with legumain, cathepsin B, or neutrophil elastase. (C) Metabolite formation from the ADCs **6a**−**c**, **e**, and a cathepsin B-cleavable control ADC after incubation with a lysosomal extract from rat liver for 48 h.

As presented in Figure 3, the excellent *in vitro* potency of ADCs **6b**, **6c**, and **6e** was fully reflected *in vivo* in the TWEAKR expressing NCI-H292 NSCLC and Ku-19-19 urothelial cancer models. Regardless of the linker chemistry, two weekly doses of these ADCs at 5 mg/kg resulted in a long-lasting NCI-H292 tumor regression, which was highly selective against the respective isotype control ADCs (Figure 3A). Similarly, high efficacy and selectivity was observed in the Ku-19-19 model with three weekly doses of the ADCs at 5 mg/kg (Figure 3B).

In conclusion, we have demonstrated that legumain-cleavable ADCs with KSP inhibitor payloads represent a versatile novel approach for cancer treatment. A remarkable tolerance of legumain for different linker peptides, including those with just a single asparagine residue, provides multiple opportunities for an adjustable linker design for the generation of ADCs. The efficient and highly specific cleavage by legumain, which is more pronounced in tumors compared to healthy organs, may provide these highly potent ADCs with an additional safety level—in addition to the antibody-mediated payload delivery. Further investigations in additional cancer indications are ongoing.

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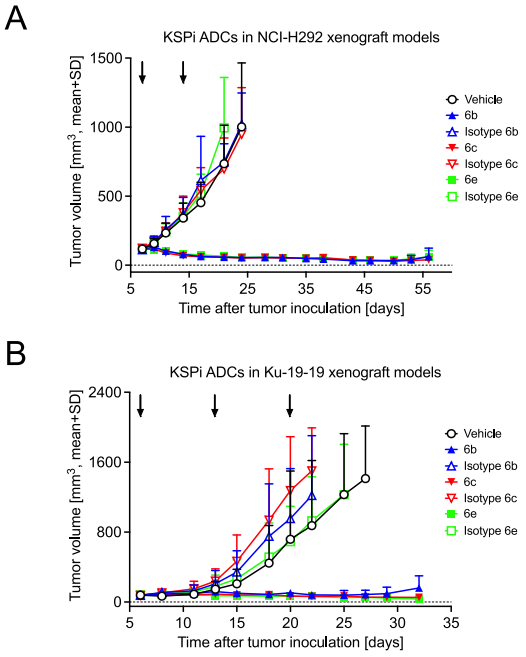

Figure 3. Efficacy of the anti-TWEAKR ADCs **6b**, **6c**, and **6e** with an antagonistic TWEAKR mAb ITEM4 and the respective isotype control ADCs in NCI-H292 (A) and Ku-19-19 (B) tumor-bearing NMRI nude mice (n = 8/group) dosed at 5 mg/kg on days 6 and 13 or on days 6, 13, and 20, respectively.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.0c00357.

Synthesis and analytical characterization of compounds, *in vitro* assays, and *in vivo* studies (PDF)

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Notes

The authors declare the following competing financial interest(s): All authors are or have been employees and are shareholders (except B.S.-L., S.G., and L.D.) of Bayer AG.

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