Design, Synthesis, In Vitro and In Vivo Evaluation of Cereblon Binding Bruton's Tyrosine Kinase (BTK) Degrader CD79b targeted Antibody Drug Conjugates

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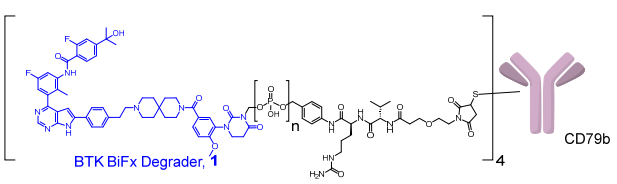
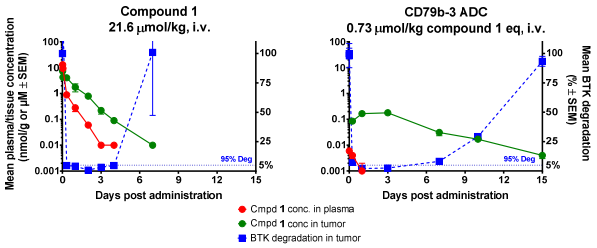


CD79b BTK BiFix Degrader ADC: n=2, **CD79b-3**
n=1, **CD79b-4**



ABSTRACT: Antibody-drug conjugates (ADCs) are an established modality which allows for targeted delivery of a potent molecule, or payload, to a desired site of action. ADCs, wherein the payload is a targeted protein degrader is an emerging area in the field. Herein we describe our efforts of delivering a Bruton's tyrosine kinase (BTK) bifunctional degrader **1** via a CD79b mAb where the degrader is linked at the ligase binding portion of the payload via a cleavable linker to the mAb. The resulting CD79b ADCs, **3** and **4**, exhibit in vitro degradation and cytotoxicity comparable to **1** and ADC **3** can achieve more sustained in vivo degradation than iv administered **1** with markedly reduced systemic exposure of the payload.

Antibody drug conjugates (ADCs) are an important modality for cancer treatment wherein a molecule with potent anticancer activity (ADC payload) is delivered via a monoclonal antibody to improve the therapeutic index[1]. There are currently thirteen clinically approved ADCs, and all are comprised of a pan cytotoxic payload molecule conjugated to a tumor targeted antibody. While the first ADC was approved in 2000, despite extensive efforts additional ADC approvals did not soon follow. However, since 2017 there have been ten new ADCs approved as aspects of ADC design and evaluation (payload, antigen, linker, conjugation technology, indication and clinical trial design) have been optimized and learnings in the field have been realized for the benefit of patients[2]. Targeted protein degradation[3–9], in which a protein of interest (POI) is tagged for degradation as a consequence of recruiting the POI to an E3 ligase in the presence of a degrader molecule, is a more recent drug modality that provides an opportunity to drug formerly undruggable targets and may offer a new approach to precedented targets. Numerous purposely designed targeted protein degraders are just now progressing through clinical development with the benefit to patients to be determined. Targeted protein degrader molecules can span a spectrum from higher molecular weight bifunctional degraders, with a POI binder and ligase binder connected by a linker, to smaller molecular glues which bind one protein partner (such as the ligase) and may have little or no binary affinity to the other. Optimizing larger bifunctional degraders for oral delivery can pose challenges given the molecular size. Depending on the POI, both bifunctional degraders and molecular glues (like other modalities) may also have challenges with respect to therapeutic index when delivered systemically. Targeted protein degrader antibody drug conjugates, with degrader molecules

serving as a new class of ADC payloads, have recently been described[10–15] as an approach to improve both the therapeutic index of targeted protein degrader molecules as well as to overcome potential PK challenges. We describe herein our efforts on targeted protein degrader ADCs, sharing how a potent Bruton's tyrosine kinase (BTK) bifunctional degrader attached to a CD79b mAb demonstrates *in vivo* degradation activity comparable to systemic administration of the parent BTK bifunctional degrader and can do so at a much lower total dose of payload molecule, resulting in a much lower systemic exposure of BTK bifunctional degrader when administered as an ADC. The chemistry connection of the bifunctional degrader to the ADC linker presented may well be applicable to other ADC payloads beyond this BTK degrader example.

Bruton's tyrosine kinase (BTK) is a cytoplasmic kinase expressed in a subset of immune cells. Aberrant BTK signaling has been associated with a variety of diseases including chronic lymphocytic leukemia, mantle cell lymphoma, systemic lupus and arthritis. Clinically approved covalent BTK kinase inhibitors such as ibrutinib[16] and acalabrutinib[17] have provided benefit for cancer patients and additional covalent and non-covalent[18] agents are in advanced clinical testing for patients with autoimmune diseases. Modulation of BTK signaling has now also been demonstrated with bifunctional degraders[19] wherein molecules binding to the kinase can recruit E3 ligases such as Cereblon (CRBN) and bring about degradation and loss of signaling. CRBN binding BTK bifunctional degrader compound **1**[20] is an example which degrades BTK and demonstrates anti-proliferative effects in the sub nM range, Figure 1 & Table 1. Consistent with the degrader mechanism, N-methylated compound **2** which disrupts CRBN binding, is significantly less active. The level of cellular potency of compound **1** is in the range of payloads often used in ADC delivery and as such we wondered whether compound **1** could be delivered via an mAb and achieve potent activity. We also wondered whether chemistry could be realized to link (and release upon ADC processing) compound **1** in a way that would be applicable to other CRBN based bifunctional degraders, especially ones that may have a narrower therapeutic index than BTK degraders.

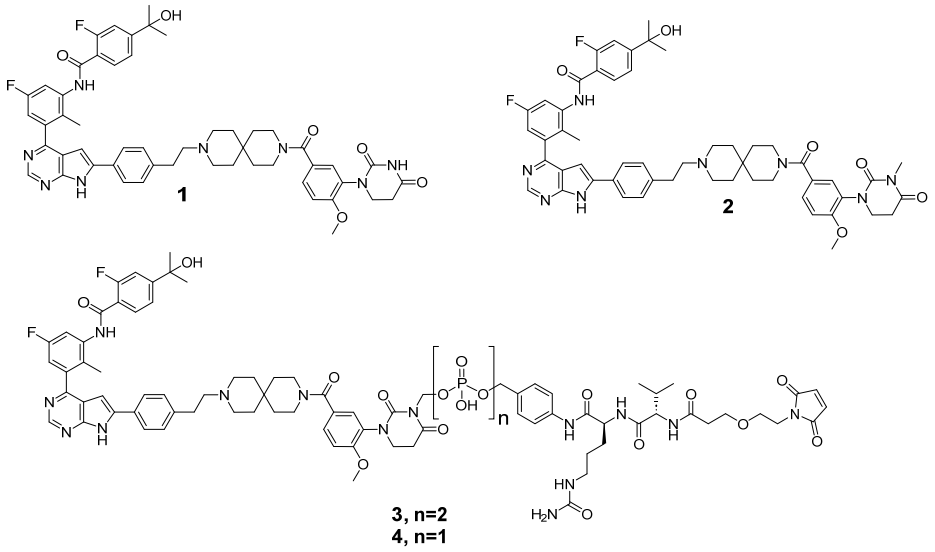


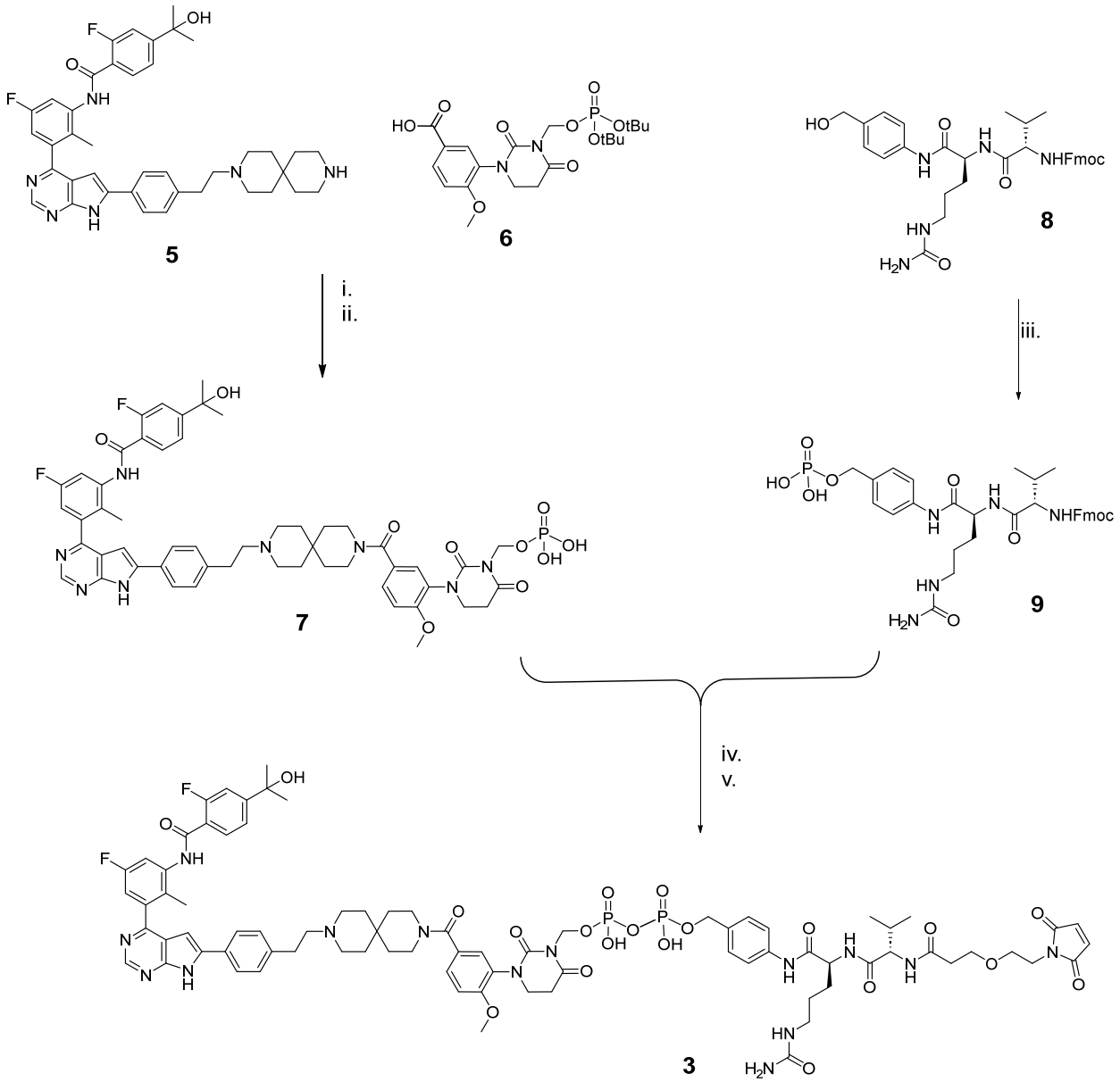
Figure 1, structure of BTK degrader, inhibitor, and ADC linker payloads

With these considerations in mind, our design focused on attaching to the dihydrouracil nitrogen in compound **1** a cleavable linker that upon ADC processing could liberate intact compound **1**. As N-imide methyleneoxy phosphates[21] and N-dihydrouracil methyleneoxy phosphates[22] have been reported as suitable prodrugs for imide like containing molecules and as glucocorticoid based ADCs have utilized pyrophosphate and phosphate linking moieties[23,24], we designed compounds **3** and **4.** These compounds both contain the clinically validated valine-citrulline para-aminobenzyl protease cleavable moiety, present in six approved ADCs, which upon proteolysis and 1,6 elimination of 4-methylenecyclohexa-2,5-dien-1-imine are designed to yield a diphosphate or monophosphate species which upon further

dephosphorylation and loss of formaldehyde then liberate compound **1**. To test our hypothesis, the syntheses of compounds **3** and 4 were pursued.

Compound **3** was synthesized as outlined in Scheme 1. Amide coupling of piperidine **5** and benzoic acid **6** under standard HATU conditions followed by TFA mediated t-butyl phosphate deprotection provided methyleneoxyphosphate **7**. Fmoc-ValCit para-aminobenzyl phosphate **9** was prepared by treating benzyl alcohol **8** with diallyl diisopropylphosphoramidite and tetrazole followed by t-butyl peroxide and then allyl deprotection under (tetrakis)triphenyl phosphine and phenylsilane conditions. The key coupling of **7** with **9** proceeded upon treatment with carbonyl diimidazole and diisopropylethylamine followed by zinc chloride. Finally, Fmoc deprotection and PEG1 Maleimide acylation of the resulting amine provided compound **3**.

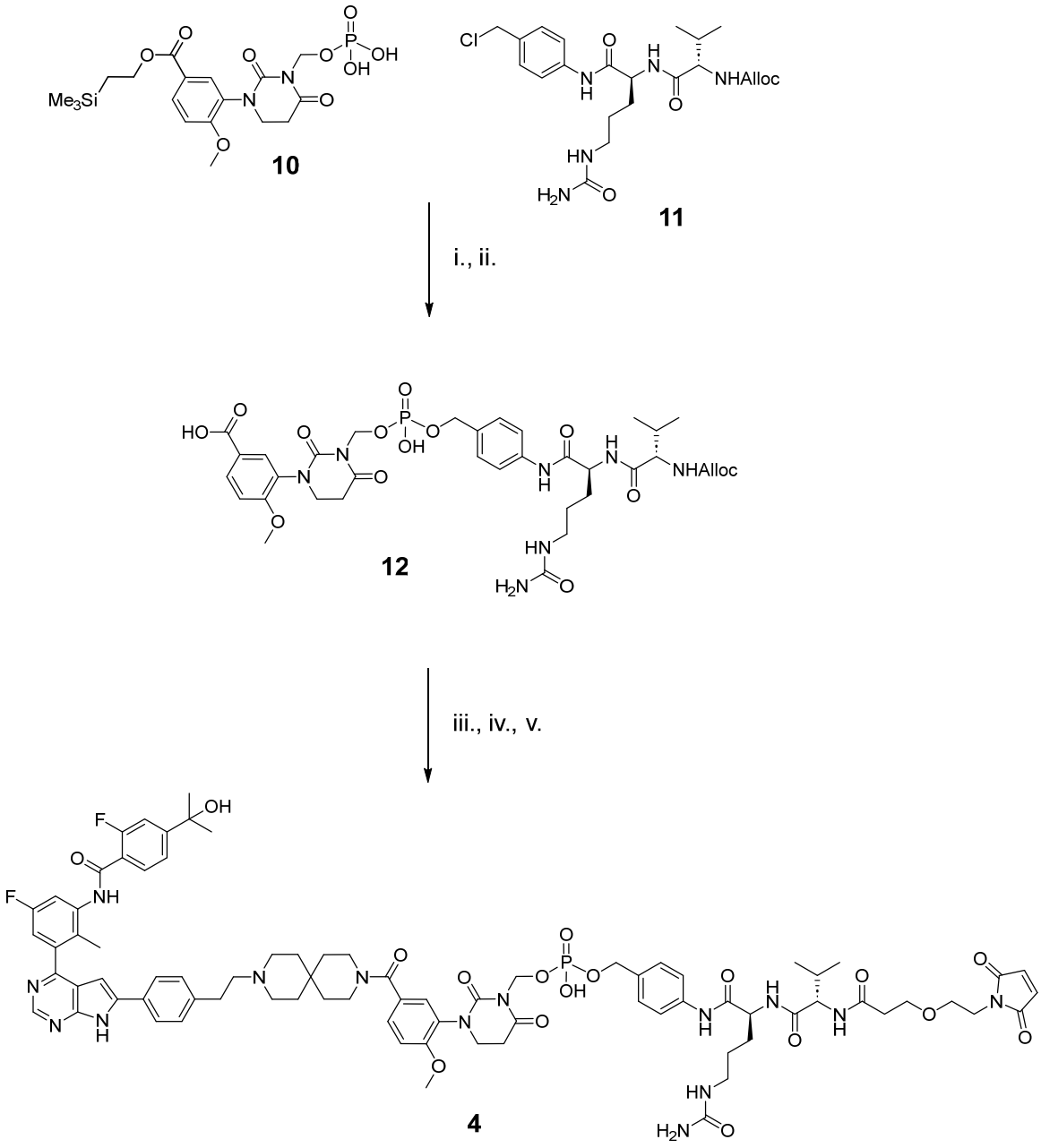
Scheme 1. Synthesis of Compound 3[a]



[a] (i) HATU, DIEA, **5**, DMF (ii) 25% TFA/CH_2Cl_2 (iii) a. $(allylO)_2PN(iPr)_2$, tetrazole, DMF b. t-BuOOH c. $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2, MeOH (iv) CDI, DIEA, DMF; $ZnCl_2$ (v) a. Piperidine/DMF b. MalPEG1NHS ester, DMF, DIEA

Compound **4** was synthesized as outlined in scheme 2. The key step was the N,N'-dicyclohexylmorpholine-4-carboximidamide (DCMC) mediated alkylation[25] of methyleneoxyphosphate **10** with AllocValCit para-aminobenzyl chloride **11** which yielded after subsequent tetrabutylammonium fluoride mediated ester deprotection phosphate ester **12**. Amide coupling of **12** with amine **5** under carbodiimide/hydroxyazobenzotriazole conditions followed by standard Alloc deprotection and PEG1 Maleimide acylation of the resulting amine provided compound **4**.

Scheme 2. Synthesis of Compound 4[a]



[a] (i) DCMC, DMF (ii) TBAF, DMF (iii) EDC, HOAT, NMM, **5**, DMF (iv) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2, MeOH (v) maleimide-PEG1-NHS ester, DIEA, DMF.

ADCs with a drug to antibody ratio (DAR) of ca. 4 were prepared by conjugation of compounds **3** and **4** to a DAR4 cysteine engineered CD79b antibody derived from the published sequence of Polatuzumab. CD79b is as well-established cell-surface-antigen and ADC target in diffuse large B-cell lymphoma and chronic lymphocytic leukemia.[26] We reasoned that following the recent front-line approval of Polatuzumab Vedotin in DLBCL that CD79b would represent an ideal model antigen for the investigation of a second-generation degrader ADC targeting B-cell malignancies.[27] A CD79b MMAE conjugate was also prepared to serve as control.

The in vitro anti-proliferative and degradation activities of the prepared ADCs were determined in the ABC-DLBCL TMD8 cell line. The potent anti-proliferative activity of the CD79b MMAE ADC confirms that CD79b delivers payload efficiently in this cell line and the lack of activity of naked CD79b mAb indicates that ADC activity is from delivered payload, Figure 2 and Table 1. The anti-proliferative activity of **CD79b-3** and **CD79b-4** was low and sub nanomolar approaching the level of activity achieved by compound **1**. A clonal TMD8 cell line engineered to express BTK fused to eGFP is a tool cell line model to determine cellular loss of BTK as measured by GFP signal in flow cytometry. **CD79b-3** and **CD79b-4** achieved sub nanomolar degradation of BTK-GFP and achieved similar activity as compared to compound **1**. The BTK Meso Scale Discovery (MSD) assay enables detection of total endogenous BTK protein in TMD8 cell lysates. **CD79b-3** and **CD79b-4** achieved equivalent degradation of endogenous BTK as compared to compound **1**. The in vitro activity of **CD79-3** and **CD79-4** relative to compound **1** indicates that a CD79b ADC can achieve targeted protein degradation leading to cell growth inhibition with single digit and sub nanomolar activity.

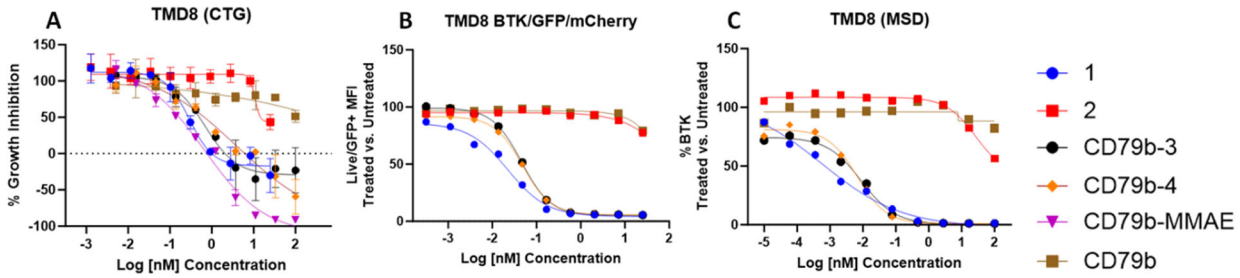


Figure 2. *in vitro* activity of small molecules and ADCs in ABC-DLBCL TMD8: growth inhibition as measured by Cell Titer-Glo (CTG) **(A)**, loss of fused BTK-eGFP in engineered cell line model measured by flow cytometry **(B)** and endogenous BTK degradation measured by MSD **(C)**

Compound	GI50 (nM, CTG)	DC50 (nM, FACS)	DC50 (nM, MSD)
1	0.290	0.022	0.0006
2	>50	>50	>50
CD79b-3	0.682	0.045	0.0089
CD79b-4	2.21	0.048	0.0067
CD79b-MMAE	0.957	n/a	n/a
CD79b	>100	>25	>25

Table 1. GI50 (nM) and DC50 (nM) values as determined by *in vitro* assays.

With promising in vitro ADC activity demonstrated, we next evaluated *in vivo* compound **1** and **CD79b-3** ADC in a PK/PD study using activated B-cell-like (ABC) DLBCL TMD8 tumor-bearing mice

(Figure 3). Compound **1** administered as a single IV injection at 20 mg/kg (21.6 µmol/kg) resulted in a Cmax in plasma of 13 µM which was cleared after 4 days. In tumor, the Cmax was slightly lower (~7 µM) but compound was still detectable 7 days post-treatment. Such exposure resulted in a full BTK degradation after 8h which was sustained for 4 days. **CD79-3** ADC administered as a single IV injection at 30 mg/kg (which contains ca. 0.71 mg/kg or 0.76 µmol/kg of compound **1**) resulted in plasma exposure of **CD79b-3** ADC within the range of expected IgGs (see Supplementary Information) and a much lower Cmax of released compound **1** in plasma (6 nM) which could be detected only for 24h. In tumor, the released compound **1** was relatively high after only 6h (0.085 µM), reached a delayed Cmax of 0.17 µM between day 1 and 3, and could be detected up to 15 days post-treatment. Despite this delayed Cmax in tumor, full BTK degradation was already reached after 6h and sustained for 6 days. From day 6 to 15, BTK went back slowly to the initial level. The significantly longer full BTK degradation induced by the released compound **1** from **CD79b-3** was consistent with the longer tumor exposure of released compound **1** achieved by ADC delivery. Such PK and PD with **CD79b-3** ADC was achieved with ~30-fold lower molar dose of compound **1**. Moreover, it translated in a 570-fold lower blood exposure of released compound **1**.

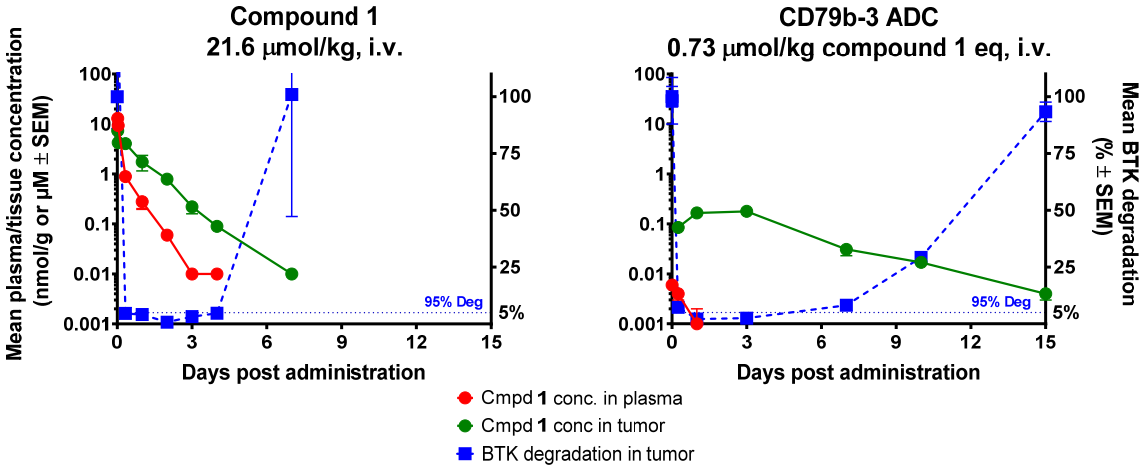


Figure 3. *In vivo* pharmacokinetics and BTK degradation of compound **1** and **CD79b-3** ADC in ABC-DLBCL TMD8 tumor-bearing mice.

In summary, we designed, synthesized, and evaluated the in vitro and in vivo properties of BTK bifunctional degrader CD79b targeting ADCs which contain a cleavable linker connecting the CRBN ligase binding portion of the degrader molecule to the mAb. The in vitro BTK degradation and anti-proliferative activities of the ADCs were comparable to the parent payload molecule. In vivo, released payload from the ADC had a flatter and longer exposure in tumor which resulted in a more sustained BTK degradation compared to that achieved from iv administration of the free parental payload. Of note, the more sustained in vivo activity of the ADC was achieved at a 30-fold lower molar dose of payload and a significantly reduced blood exposure of the released payload molecule. Finally, we anticipate the linker chemistry described to be applicable to other ADC payloads.

Methods and Experimental Details are available in the Supplementary Information.

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