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Letter

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Nicotinamide Phosphoribosyltransferase Inhibitor as a Novel Payload for Antibody−Drug Conjugates

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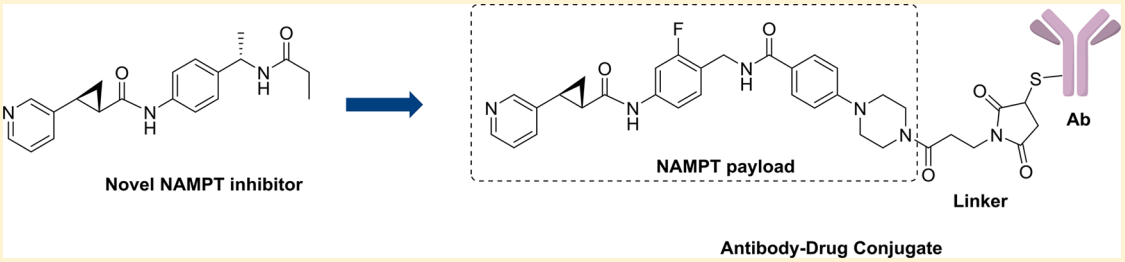
Supporting Information



ABSTRACT: Antibody−drug conjugates (ADCs) are a novel modality that allows targeted delivery of potent therapeutic agents to the desired site. Herein we report our discovery of NAMPT inhibitors as a novel nonantimitotic payload for ADCs. The resulting anti-c-Kit conjugates (**ADC-3** and **ADC-4**) demonstrated *in vivo* efficacy in the c-Kit positive gastrointestinal stromal tumor GIST-T1 xenograft model in a target-dependent manner.

KEYWORDS: *Antibody−drug conjugate (ADC), nicotinamide phosphoribosyltransferase (NAMPT), novel payload*

Antibody−drug conjugates (ADCs) aim to deliver potent therapeutic agents to desired pathological sites while sparing healthy tissues from deleterious side effects.[1] Of all conjugates currently undergoing clinical evaluation, those within the oncology space largely predominate. This includes four clinically approved ADCs Mylotarg,[2] Adcetris,[3] Kadcyla,[4] and Besponsa.[5] Despite this obvious bias, there are also efforts to extend the scope of this powerful technology toward targeting immune cells[6] or treating bacterial infections.[7] ADCs generally comprise three key components: an antibody, a linker, and a drug (payload). An antibody component, which serves as a targeting ligand, would typically recognize a receptor that is highly and selectively expressed on the cells of interest. Cleavable or noncleavable linkers represent the second key element of ADCs. Cleavable linkers are designed to release a free intact parent drug,[8] which could exert a subsequent bystander killing, whereas noncleavable linkers result in formation of a charged adduct derived from the antibody catabolism in lysosomal compartments.[9] To date, the choice of payload utilized in the ADC approach has been limited. Of the four approved ADCs, two (Adcetris and Kadcyla) employ tubulin-binding payloads (auristatines and maytansines) and the other two use the DNA double strand breaking compound calicheamicin. A literature survey reveals that only a few alternative mechanisms-of-action (MoAs) have been studied in the ADC context clinically. These include DNA minor groove binders pyrrolobenzodiazepines,[10] a TOPO1 inhibitor camptothecin,[11] and DNA alkylators such as indolinobenzodiazepines[12] and duocarmycins.[13]

Screening of various potential MoAs in a select panel of cell lines, including c-Kit and HER2 expressing lines (NCI-H526, MDA-MB453, and NCI-N87) identified the NAMPT inhibitor **1** as being potent across multiple cell lines in the nanomolar range (Figure 1A, Table 1). Identification of cell lines both sensitive to the payload MoA and expressing the target receptor was a critical criterion in the selection of a new MoA for ADC delivery.

The NAMPT enzyme converts nicotinamide to nicotinamide mononucleotide and is the rate-limiting enzyme that controls intracellular NAD^+ concentration.[14] The efficacy of NAMPT inhibitors has been demonstrated in various preclinical settings and several NAMPT inhibitors, e.g., **APO866 (FK866)**,[15] **GMX1778**, and **GMX1777**,[16] advanced into the clinic (Figure 1B).

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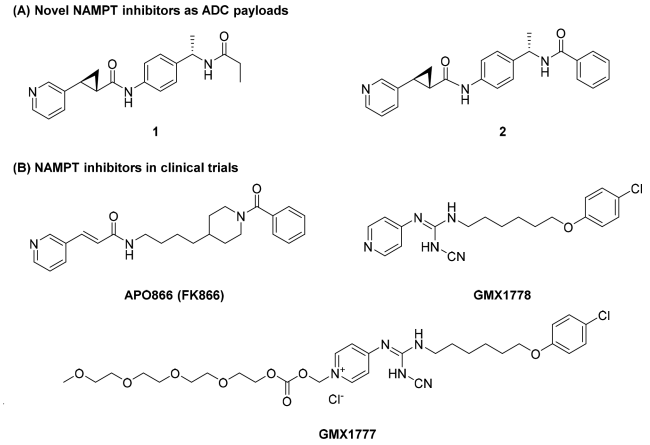


Figure 1. (A) Novel NAMPT inhibitors as ADC payloads and (B) NAMPT inhibitors in clinical trials.

Because clinical utility has been limited by on-target and dose-limiting toxicities such as thrombocytopenia and GI effects, we envisioned that vectorized, targeted delivery of NAMPT inhibitors into tumor tissues via an ADC route might significantly improve their therapeutic index.[17]

Compounds **1** and **2** originated from an internal research effort to discover novel NAMPT inhibitors.[18] This gave us a solid platform with which we could begin our evaluation of NAMPT inhibitors as a novel ADC payload. We reasoned that **1** would be an excellent starting point for our optimization efforts due to the availability of X-ray cocrystal information with the NAMPT enzyme (Figure 2, PDB code 1gqh).[18] This would help guide our design toward maximizing the payload potency and determine suitable exit vectors for our linkers.

Before embarking on our exploration, we had to take into account a key aspect of the NAMPT inhibitor. The pyridine nitrogen is known to undergo ribophosphorylation by the NAMPT enzyme and is thus an essential part of the pharmacophore. Furthermore, we reported that the whole 3-pyridine (*S*,*S*) cyclopropyl carboxamide motif is crucial to achieve the highest level of cellular activity; therefore, we did not pursue further optimization of this portion of the molecule. Instead, we performed a focused SAR exploration in the R_3 region, which points toward a solvent exposed area. As shown previously, various substituents are tolerated at this position. In

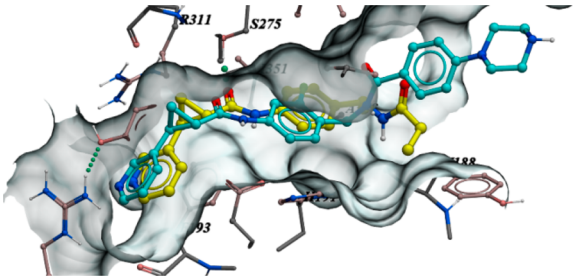
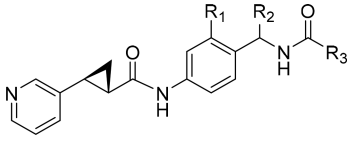


Figure 2. Overlay of the X-ray structure of **1** cocrystallized with the NAMPT enzyme (yellow, PDB code 1gqh) and **4** docked at the binding site (cyan).

particular, the ethyl substituent can be replaced by a more rigid unsubstituted phenyl ring (**2**, Table 1). This substitution resulted in a comparable cellular potency when tested on the NAMPT sensitive A2780 and CORL23 cell lines. As our ultimate goal was to generate conjugates, we also included the cell lines NCI-H526, MDA-MB453, and NCI-N87 in our panel. NCI-H526 expresses high levels of the receptor tyrosine kinase c-Kit, whereas MDA-MB453 and NCI-N87 cell lines over-express the receptor tyrosine kinase HER2, both suitable antigens for ADC targeting.

Molecular docking suggested that the phenyl ring can be further derivatized at the para-position providing an optimal vector for linker attachment. Next, we needed to introduce an appropriate functional group that could be utilized for linker attachment. Although many functional groups have been employed in the context of ADCs, secondary or primary amines offer the highest flexibility and can be modified with both cleavable and noncleavable linkers. Therefore, our goal was to append a primary or secondary amine to the phenyl ring while retaining or improving the payload potency. Among various groups that have been tried, a piperazine substituent installed at the para-position of the phenyl ring turned out to be well tolerated (**3**, Table 1). Furthermore, molecular docking suggested that the free secondary amine on the piperazine group points into a solvent-exposed area and can be utilized as a handle for linker attachment to allow subsequent conjugation to an antibody. Phenylpiperazine became our preferred substituent at position R_3. Finally, keeping R_3 constant, we revisited the SAR in the central portion of the payload. A quick evaluation of R_1

Table 1. Cellular Profiling of NAMPT Payloads



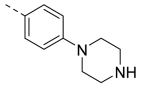
Compound	R_1	R_2	R_3	A2780 (nM)	CORL23 (nM)	NCI-H526 (c-Kit+, nM)	MDA-MB453 (HER2+, nM)	NCI-N87 (HER2+, nM)
1	H	(*S*)-CH_3	CH_3CH_2	5	18	32	17	14
2	H	(*S*)-CH_3	Ph	1	14	11	2	3
3	H	(*S*)-CH_3		7	7	11	1	1
4	F	H		5	19	2	0.4	1
5	H	H		22	64	31	4	10

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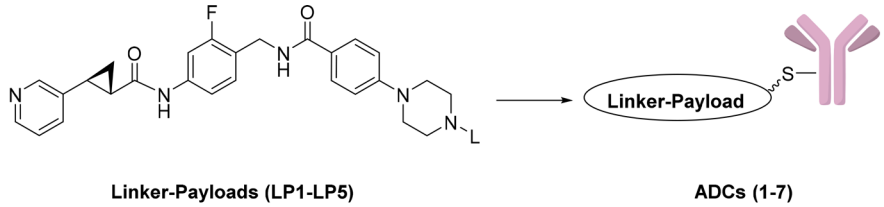
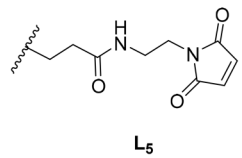
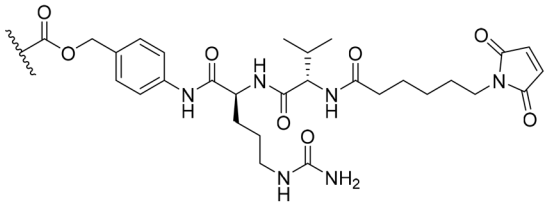
Table 2. ***In Vitro*** **Cytotoxicity of NAMPT ADCs**

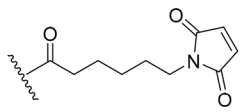




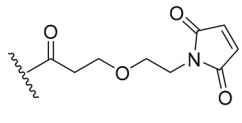
L_5



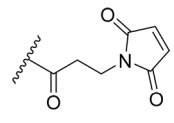
L_1



L_2



L_3



L_4

ADC	linker-payload	linker (L)	target antigen	Aggr. (%)	DAR	GIST-T1 (c-Kit$^+$, nM)	NCI-H526 (c-Kit$^+$, nM)	MDA-MB453 (HER2$^+$, nM)	NCI-N87 (HER2$^+$, nM)
ADC-1	**LP1**	L_1	c-Kit	29	2.8	0.014	>0.720	not tested	not tested
ADC-2	**LP2**	L_2	c-Kit	23	4.0	<0.003	0.047	>33	>33
ADC-3	**LP3**	L_3	c-Kit	6.1	3.9	<0.003	0.009	2.13	4.05
ADC-4	**LP4**	L_4	c-Kit	5.3	2.3	<0.007	0.040	6	12
ADC-5	**LP4**	L_4	HER2	6.2	2.9	18.7	71.5	0.006	0.017
ADC-6	**LP4**	L_4	IgG	10	4.4	0.1274	12.5	1.42	2.76
ADC-7	**LP5**	L_5	c-Kit	23	3.9	<0.003	0.352	>33	>33

and R_2 substituents resulted in a slightly improved analogue **4** (the payload synthesis and characterization is described in the Supporting Information).

Payload **4** displayed single digit nanomolar potency on c-Kit and HER2 expressing cell lines and was, therefore, selected for a subsequent modification with a focused set of cleavable (L_1) and noncleavable (L_2–L_5) linkers (Table 2). All linker-payloads were initially conjugated to an anti-c-Kit monoclonal antibody selected for its ability to efficiently internalize in c-Kit expressing cells. Maleimide chemistry was used to conjugate the linker-payloads to interchain disulfides of the antibodies (the linker-payload synthesis and their characterization and bioconjugation methods are described in the Supporting Information). The cathepsin-cleavable ValCit linker L_1, which has been widely used in the literature and utilized in the approved ADC Adcetris,[3] was our first choice, as it should furnish the unmodified payload **4** upon intracellular release. Despite the promising *in vitro* potency of the conjugate **ADC-1**, the utility of this linker is compromised by the resulting high aggregation of 29% (Table 2). Therefore, we turned our attention to the noncleavable linkers (L_2–L_5). It is worth mentioning that, while linkers L_2–L_4 turn the secondary amine of the payload into an amide, the L_5 linker was designed to retain the basic tertiary amine, which we thought would be the closest version of payload **4**. Interestingly, linker L_5 along with linker L_2 resulted in conjugates with high aggregation. In contrast, linkers L_3 and L_4 reduced aggregation to the acceptable level of below 10% and afforded drug-to-antibody ratio (DAR) in the range of 2–4. All conjugates were tested for *in vitro* cytotoxicity on c-Kit expressing cell lines such as GIST-T1 and NCI-H526. The anti-cKIT conjugates **ADC-3** and **ADC-4** turned out to be not only low aggregating but also possessed the best potency (IC_{50} of 9 and 40 pM, respectively) on the NCI-H526 line, representing a lower antigen density compared to GIST-T1. As a consequence, two linker-payloads (**LP3** and **LP4**) emerged as our leads. This led to conjugation of **LP4** to an anti-HER2 trastuzumab (**ADC-5**) and a nonbinding isotype control antibody (**ADC-6**). The target-dependent cellular activity was confirmed (Table 2), with the nonbinding IgG isotype control **ADC-6** being significantly less active.

Next, we confirmed the mechanism-dependent pharmacodynamic (PD) modulation by looking at the NAD$^+$/NADH levels upon exposure of the GIST-T1 cell line to our anti-c-Kit conjugate **ADC-4** in comparison to the nonbinding control **ADC-6** (Figure 3). To this end we employed a cellular assay

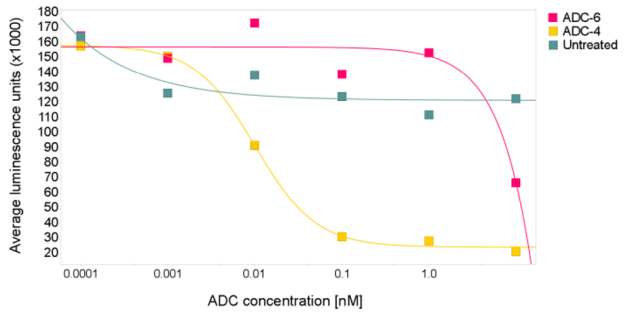


Figure 3. Mechanism-dependent PD modulation: NAD$^+$/NADH levels in GIST-T1 cells at 24 h post-dosing.

system that uses a luminescent substrate to quantify cellular NAD$^+$/NADH levels and compared the activity of **ADC-4** to that of the nonbinding **ADC-6**, demonstrating that only the c-Kit-targeting **ADC-4** resulted in a concentration-dependent inhibition of signal.

In addition, the nonantimitotic MoA of our lead conjugate was confirmed by analyzing the cell-cycle profiles of GIST-T1 cells exposed to either s-methyl-DM1 (s-me-DM1), a cell-permeable maytansine analog,[19] or **ADC-4**.

Treatment with s-me-DM1 resulted in accumulation of cells in G2/M phase, consistent with inducing cell cycle arrest at the

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G2/M transition. This was due to microtubule depolymerization. In contrast, treatment of GIST-T1 cells with **ADC-4** inhibited cell proliferation (Figure 4B) without overtly affecting the cell cycle (Figure 4A).

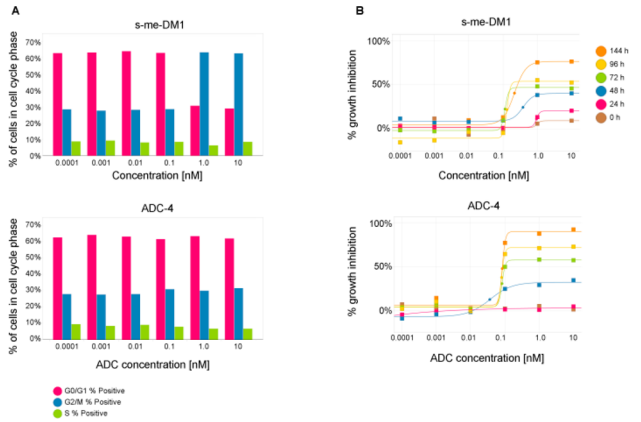

Figure 4. (A) Cell cycle and (B) kinetic viability analysis in GIST-T1 cells treated with either s-me-DM1 or **ADC-4**.

Encouraged by the *in vitro* activity, the anti-c-Kit **ADC-3** and **ADC-4** were assessed for *in vivo* efficacy in the c-Kit positive gastrointestinal stromal tumor GIST-T1 xenograft model in mice. A single 20 mg/kg administration of either anti-cKit ADCs induced tumor stasis in the GIST-T1 model, with a clear differential from the nonbinding isotype control **ADC-6** treated group (Figure 5A).

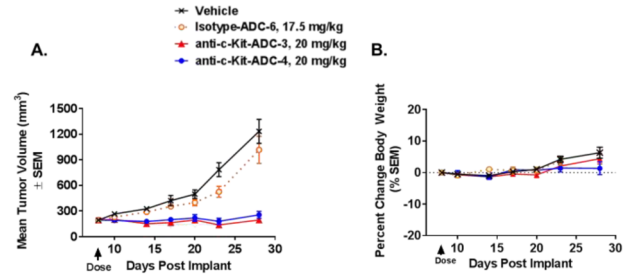

Figure 5. (A) Antitumor efficacy after a single intravenous administration of vehicle, isotype control **ADC-6**, or anti-c-Kit **ADC-3** or **ADC-4** at indicated dose levels in the GIST-T1 xenograft model in SCID bg mice and (B) effect on the body weight.

The conjugates were well tolerated at this dose as indicated by the stable body weight (Figure 5B).

In summary, we designed and synthesized a series of antibody−drug conjugates employing payloads with a novel NAMPT inhibitor mechanism of action. The two noncleavable linker formats afforded ADCs that were generally low-aggregating with highly promising *in vitro* profiles. Furthermore, the conjugates were well tolerated and demonstrated target-dependent efficacy *in vivo*. The conjugates are currently undergoing further evaluation across additional *in vivo* models.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00254.

Complete experimental procedures and analytical characterization of novel compounds, bioconjugation methods, *in vitro* cytotoxicity, and *in vivo* efficacy data (PDF)

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The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ADC, antibody−drug conjugate; Aggr, aggregation; DAR, drug-to-antibody ratio; MoA, mechanism-of-action; NAD+, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); NAMPT, nicotinamide phosphoribosyltransferase; NMN, nicotinamide mononucleotide; RNA, ribonucleic acid

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