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Integrin-Targeting Knottin Peptide–Drug Conjugates Are Potent Inhibitors of Tumor Cell Proliferation

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Abstract: *Antibody–drug conjugates (ADCs) offer increased efficacy and reduced toxicity compared to systemic chemotherapy. Less attention has been paid to peptide–drug delivery, which has the potential for increased tumor penetration and facile synthesis. We report a knottin peptide–drug conjugate (KDC) and demonstrate that it can selectively deliver gemcitabine to malignant cells expressing tumor-associated integrins. This KDC binds to tumor cells with low-nanomolar affinity, is internalized by an integrin-mediated process, releases its payload intracellularly, and is a highly potent inhibitor of brain, breast, ovarian, and pancreatic cancer cell lines. Notably, these features enable this KDC to bypass a gemcitabine-resistance mechanism found in pancreatic cancer cells. This work expands the therapeutic relevance of knottin peptides to include targeted drug delivery, and further motivates efforts to expand the drug-conjugate toolkit to include non-antibody protein scaffolds.*

Cystine-knot mini-proteins or "knottins" are short (30–50 amino acid) peptides[1] that possess many therapeutically desirable characteristics.[2] These include high thermal and proteolytic stability and the potential for oral administration;[3] features resulting from a highly compact cystine-knot framework. As a result, several knottins have been investigated for clinical applications,[4] including treatments for cancer,[5] chronic pain,[6] wasting syndrome,[7] and as tumor-imaging agents.[8]

We have developed an engineered knottin, EETI-2.5F (**1**), which has been evolved to target a panel of integrin receptors (including αvβ3, αvβ5, and α5β1) that are highly-expressed in many types of cancer.[9] Owing to the role of integrins in cellular proliferation, migration, angiogenesis,[10] and adhesion to the extracellular matrix,[11] elevated integrin expression plays a critical role in tumor progression.[12] As a result, integrins have received significant attention as potential targets in cancer intervention.[12,13]

Previously, we showed that when labeled with fluorescent small molecules,[14] radioisotopes,[14,15] or ultrasound contrast reagents,[16] **1** can be used for the in vivo imaging of tumors in mice, including intracranial patient-derived xenografts[8b,c] (Figure 1 A). These studies demonstrated high tumor imaging contrast with low levels of probe accumulation in non-target organs. We reasoned that **1** might be leveraged to deliver a drug payload selectively to tumors; a highly desirable goal as evidenced by significant investment in the development of ADCs over the past 60 years.[17]

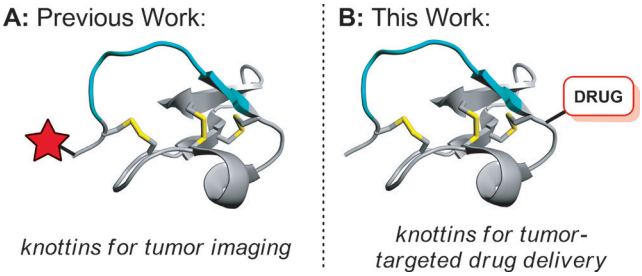

Figure 1. A) Previous work: knottins labeled with fluorescent small molecules, radioisotopes, and ultrasound contrast reagents for tumor-imaging. B) This work: knottin peptide–drug conjugates (KDCs) for tumor-targeted drug delivery.

In this work (Figure 1 B), we describe a series of knottin peptide–drug conjugates, synthesized using a variety of drug-linker strategies, and highlight an optimal conjugate as a potent inhibitor of tumor cell growth in vitro against a variety of malignant cell lines. We provide evidence that: 1) integrin-binding is essential for potency, 2) the mechanism of cellular internalization is integrin-mediated, and 3) the drug payload is released intracellularly. We envisioned that a variant of knottin EETI-2.5F (**1**) containing an azide-bearing unnatural amino acid would allow for efficient preparation of drug conjugates through azide–alkyne cycloaddition (Figure 2). In support of this strategy, we recently described a version of EETI-2.5F that tolerated the substitution of an unnatural amino acid at position 15.[18] We therefore prepared the azido-variant EETI-2.5Z (**2**) using solid-phase peptide synthesis (Figures S1, S2, and Tables S1, S2), and showed that it retained low-nanomolar binding affinity to U87MG glioblastoma cells (Figure 2).

We next sought a cytotoxic payload that could be efficiently conjugated to **2**. We identified gemcitabine (**3**)[19] as a candidate given its precedence as a widely-used chemo-

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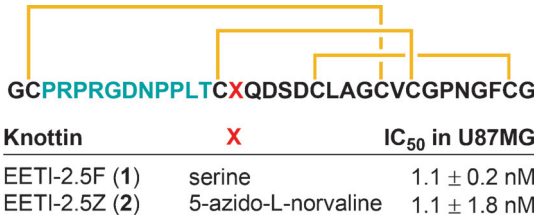


Figure 2. Sequence of EETI-2.5F (**1**) and EETI-2.5Z (**2**) with integrin-binding loop highlighted in blue and disulfide linkages of the cystine-knot scaffold depicted in yellow. Position 15 (red X) indicates the site where an azide-containing unnatural amino acid, 5-azido-L-norvaline, was installed to allow for site-specific bioconjugation of linker–drug constructs. Substitution at this position does not disrupt binding to U87MG cells, as indicated by the similar binding constants obtained for EETI-2.5F and EETI-2.5Z.

Table 1: Binding affinity (IC_{50}) and potency (ED_{50}) in U87MG cells.

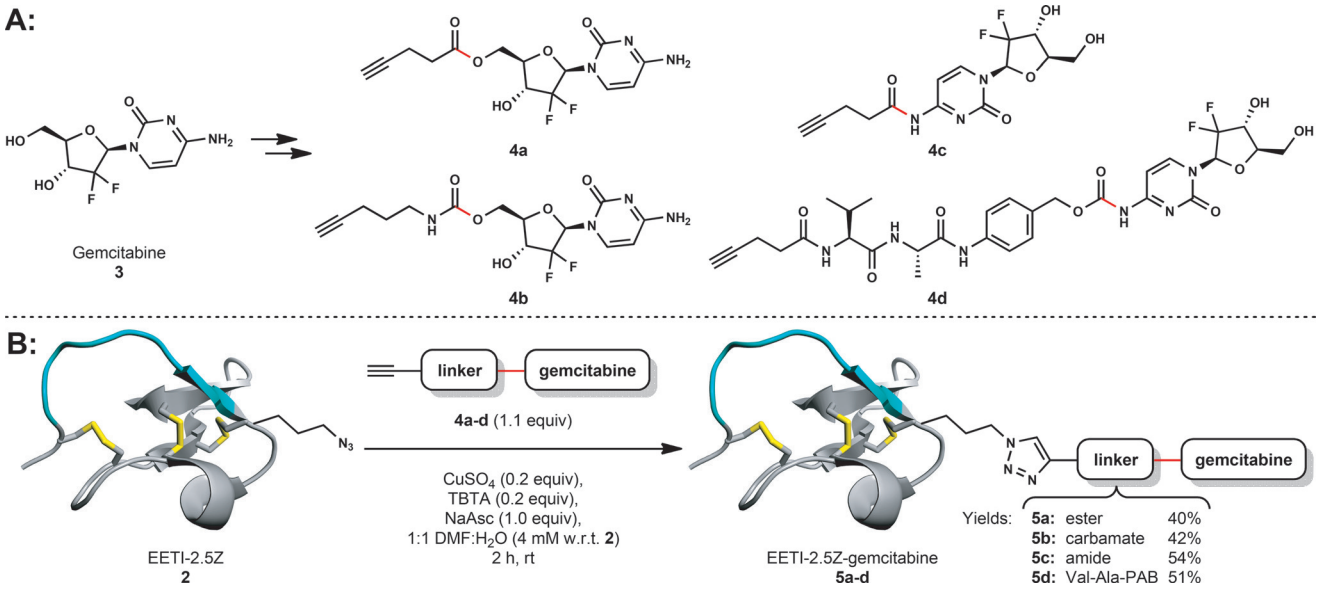
	Compound	Description	IC_{50} [nM][a]	ED_{50} [nM][b]
1	**2**	EETI-2.5Z w/o drug	1.1 ± 1.8	> 1,000
2	**5a**	ester linker	5.2 ± 3.6	8.5 ± 3.3
3	**5b**	carbamate linker	3.3 ± 0.2	> 1,000
4	**5c**	amide linker	2.8 ± 0.2	8.9 ± 1.2
5	**5d**	Val-Ala-PAB linker	1.5 ± 0.2	9.0 ± 1.8
6	**3**	gemcitabine	N/A	1.1 ± 1.4

[a] Cells bound by AF488-labeled **1** were titrated with each compound and the percent bound was measured by flow cytometry. [b] Cell proliferation was quantified 4 d after treatment with each compound using CCK-8 colorimetric assays and compared to the untreated control. Dose response curves representing competition binding and inhibition of cell proliferation are shown in Figures S7 and S8, respectively.

therapeutic,[20] its high potency against malignant cells,[21] and its tractable derivatization from inexpensive starting materials. We anticipated that linker stability would be a critical design consideration. Ideally, the linker will remain stable in the extracellular environment and release its payload only upon internalization. We therefore prepared alkyne-bearing gemcitabine derivatives tethered through several functional groups, including an ester (**4a**), a carbamate (**4b**), and an amide (**4c**). Additionally, given the extensive use of dipeptide-based cleavable linkers in ADCs,[17c] we prepared a Val-Ala-PAB (valyl-alanyl-*para*-aminobenzyloxy) derivative (**4d**) that employs a linker known to be extracellularly stable but is cleaved upon internalization by proteases such as cathepsin B.[22] Each gemcitabine derivative was then linked to EETI-2.5Z by copper-catalyzed azide–alkyne cycloaddition[23] (Scheme 1 B) to afford KDCs (**5a–d**).

Once the KDCs **5a–d** were prepared, we measured their binding affinity to U87MG glioblastoma cells, which have elevated expression of tumor-associated integrins.[24] All of the KDCs tested bound to U87MG cells with low-nanomolar affinity (Table 1), indicating that the presence of the linker and drug do not interfere with tumor targeting by the knottin. Next, we tested the potency of each KDC in a cell-proliferation experiment. KDCs with linkers containing the ester (**5a**), amide (**5c**), and Val-Ala-PAB (**5d**) moieties demonstrated low-nanomolar ED_{50} values in U87MG cells, similar to unconjugated gemcitabine (**3**). In contrast, EETI-2.5Z (**2**) was not potent, indicating that the conjugation of **3** is necessary for growth inhibition. The KDC containing the carbamate linker (**5b**) also lacked significant potency, which can be explained by the greater stability of its linker[25] and the requirement of linker cleavage in order for the payload to become active.[26]

We next sought to determine the relative extent of premature drug-release in order to identify the linker best-suited for delivering the drug payload intracellularly. We employed an established technique for blocking the cellular uptake of gemcitabine using *S*-(4-nitrobenzyl)-6-thioinosine (**6**), which inhibits nucleoside transporters such as the human Equilibrative Nucleoside Transporter 1 (hENT1).[27] Because hENT1 is a critical regulator of the cellular internalization of



Scheme 1. A) Synthesis of alkyne-bearing gemcitabine derivatives **4a–d** containing cleavable linkers. Bonds highlighted in red indicate likely sites for drug cleavage to release gemcitabine. B) Conjugation of compounds **4a–d** to EETI-2.5Z by Cu-catalyzed azide–alkyne cycloaddition, affording KDCs **5a–d**. Synthesis and characterization of these compounds are shown in Figures S3–S6 and Tables S3–S6.

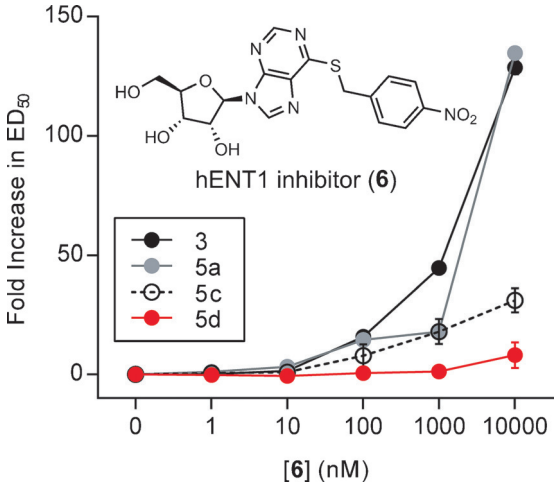


Figure 3. KDC **5d** demonstrates minimal extracellular drug-release. Blocking U87MG cellular uptake of gemcitabine **3** (black) with hENT1 inhibitor **6** significantly diminishes its potency. This effect is also observed with KDCs **5a** (gray) and **5c** (dashed), indicating significant extracellular drug-release. Meanwhile, the ED_{50} of **5d** (red) is insensitive to **6**, indicating little premature drug-release. Representative dose response curves are shown in Figures S9 and S10. ED50 values are given in Table S7, and fold increase values in Table S8.

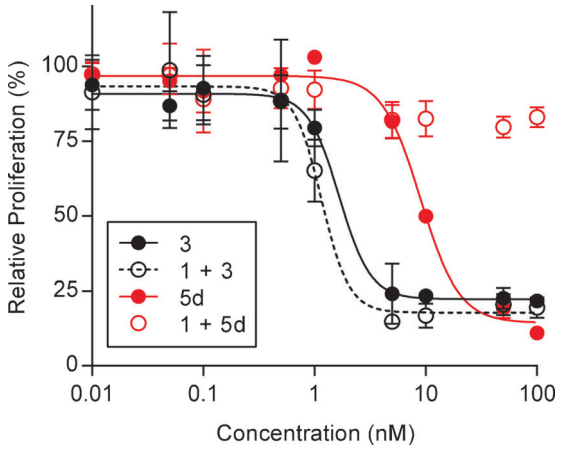


Figure 4. Integrin-binding is essential for the potency of KDC **5d**. Blocking integrins on the surface of U87MG cells by competition with 1 μM of unlabeled knottin **1** significantly reduces the potency of KDC **5d** (red), indicating that integrin-binding is critical for inhibition of tumor cell growth. Meanwhile, integrin blocking by **1** does not diminish the potency of gemcitabine **3** (black).

3,[28] by inhibiting its activity with **6**, the cytotoxic effect of any drug released extracellularly would be diminished. We anticipated that this would allow us to differentiate KDCs that were potent owing to the undesirable release of **3** outside of the cell (Supporting Information, Scheme S10). As shown in Figure 3, the ED_{50} values of unconjugated **3** as well as KDC **5a** in U87MG cells increased significantly when the cells were treated with increasing concentrations of **6**. These results indicate that in the case of **5a**, a significant amount of the linker is cleaved prematurely outside of the cell, and that the released payload **3** is still reliant upon hENT1 activity for cellular uptake. However, in the case of **5c** and **5d**, we found that potency was much less sensitive to the concentration of **6**. In particular, the potency of EETI-2.5Z-Val-Ala-PAB-gemcitabine (**5d**) was only slightly diminished even when the highest dose of **6** (10 μM) was used. This suggests that the mechanism of internalization of the drug payload in the case of **5d** is independent of hENT1, indicative of minimal premature drug release.

To determine the importance of integrin-binding for the potency of each KDC, we carried out a competition experiment in which U87MG cells were pre-treated with unconjugated EETI-2.5F (**1**), thereby blocking integrins on the cell surface. In agreement with the hENT1 inhibition experiment, we found that pre-treatment with **1** did not diminish the potency of **5a** and **5c** (Figures S11, S12). This suggests that for these KDCs, integrin binding is not essential for internalization of the payload, consistent with a case where potency results primarily from premature drug release. In stark contrast, we found that pre-treatment of U87MG cells with **1** abolished the potency of KDC **5d**, even at the highest concentration tested (Figure 4). These results suggest that for **5d**, integrin-binding is a critical part of the mechanism by which it delivers its cytotoxic payload, consistent with integrin-mediated internalization followed by intracellular drug-release. Taken together, the results shown in Table 1 and Figures 3 and 4 suggest that KDC **5d**: 1) targets cells based on the expression of tumor-associated integrins, 2) is internalized by a mechanism that relies upon integrin-binding, and 3) releases its drug payload in significant amounts only upon intracellular degradation of the linker, likely by lysosomal proteases such as cathepsin B.[22]

Having identified KDC **5d** as a potent inhibitor of U87MG cells with desirable payload release properties, we next tested its efficacy against a variety of other cell lines in comparison with gemcitabine (**3**). As shown in Table 2 (entries 2–5), **5d** is also a highly potent inhibitor of D270 glioblastoma, MDA-MB-468 breast cancer, A2780 ovarian cancer, and BxPC3 pancreatic cancer cell lines. The high potency of **5d** against PANC-1 pancreatic cancer cells (Table 2, entry 6) is particularly noteworthy given the high resistance of this cell line to gemcitabine, which is thought to be associated with its diminished nucleoside transporter activity.[29] Our data suggests that the significantly higher potency of **5d** against this cell line results from an alternative, integrin-mediated pathway for cellular uptake, obviating reliance on transporters such as hENT1.

In conclusion, we describe an engineered knottin peptide–drug conjugate, EETI-2.5Z-Val-Ala-PAB-gemcitabine (**5d**),

Table 2: Cell growth inhibition by KDC (**5d**) vs. gemcitabine (**3**).[a]

	Cell Line	Cancer Type	**5d** ED_{50} (nM)	**3** ED_{50} (nM)
1	U87MG	glioblastoma	9.0 ± 1.8	1.7 ± 1.4
2	D270	glioblastoma	7.9 ± 0.8	1.5 ± 0.4
3	MB-468	breast	0.6 ± 0.1	10.3 ± 6.8
4	A2780	ovarian	2.3 ± 0.5	0.5 ± 0.02
5	BxPC3	pancreatic	1.8 ± 0.8	18.4 ± 1.3
6	PANC-1	pancreatic	2.1 ± 0.2	52.8 ± 4.9

[a] Cell proliferation was quantified 4 d after treatment with **5d** or **3** using CCK-8 colorimetric assays and compared to the untreated control. Dose response curves for these data are presented in Figures S13–S17. Metabolic activity measured by CCK-8 was validated by quantifying cell death using Trypan Blue in Figure S18.

and show that it is a highly potent inhibitor of tumor growth in a broad panel of malignant cell lines. Our results show that this KDC targets malignant cells by binding selectively to tumor-associated integrins, and that release of its cytotoxic payload is governed by intracellular processes. Overall, these results motivate further development and testing of KDCs for applications in cancer therapy. In addition, this work supports strategies relying upon tumor-associated integrins as targets for drug delivery, and validates antibody-alternatives as scaffolds for drug conjugation.

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