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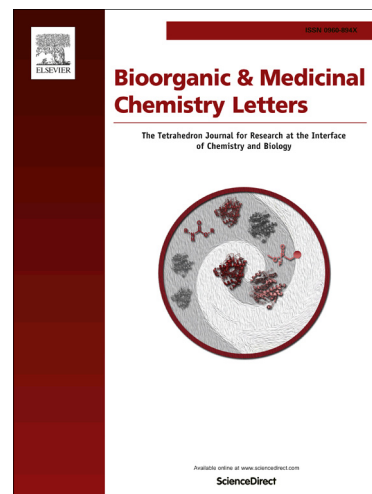
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The Cryptophycins as Potent Payloads for Antibody Drug Conjugates

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The cryptophycins are a potent class of cytotoxic agents that were evaluated as antibody drug conjugate (ADC) payloads. Free cryptophycin analog **1** displayed cell activity an order of magnitude more potent than approved ADC payloads MMAE and DM1. This potency increase was also reflected in the activity of the cryptophycin ADCs, attached via a either cleavable or non-cleavable linker.

Antibody drug conjugates (ADCs) are currently an emerging class of treatment for targeted therapies against cancer.¹⁻⁴ ADCs consist of an antibody conjugated to a potent cytotoxic agent that targets a specific antigen overexpressed on a tumor cell. The antibody directs the ADC to the preferred site of action and, following internalization, the cytotoxin is released, causing cell death. The recent approvals of ADCETRIS® (brentuximab vedotin)⁵ and KADCYLA® (ado-trastuzumab emtansine)^{6,7} have heralded in this new class of therapeutics with the potential to transform cancer treatments. As an indication of this promise, over 30 ADCs are currently in various clinical trials.^{1,8}

However, the vast majority of clinical (and approved) ADCs utilize two payloads, auristatin and maytansine derivatives.⁹ While the use of these cytotoxic agents has proven successful, the next generation of ADCs has the opportunity to make use of additional payloads with the potential to address current shortcomings such as selectivity, resistance, potency and undesirable physiochemical properties, amongst others.

The cryptophycins (Figure 1) were isolated from cyanobacteria of the genus *Nostoc* in the early 1990s and displayed potent activity against cancer cell lines.¹⁰ Subsequently, they were found to bind to microtubules at the *vinca* binding site.¹¹⁻¹³ Significant efforts were put forth to advance this class of compounds clinically but results from clinical trials indicated unacceptable

levels of toxicity at doses required for a therapeutic effect.^{14,15} Although the cryptophycins were unable to advance as stand-alone agents, they possess characteristics that make them suitable as ADC payloads: a high level of potency, relative hydrophilicity (Figure 1), and lack of Pgp susceptibility,¹⁶ a common resistance mechanism for ADCs.¹⁷⁻¹⁹ Additionally, while cryptophycin itself lacks a suitable chemical handle for attachment to an antibody, analogs have been described²⁰⁻²² that retain potency and incorporate a functional²³ handle in the form of a nucleophilic amine (Figure 1).

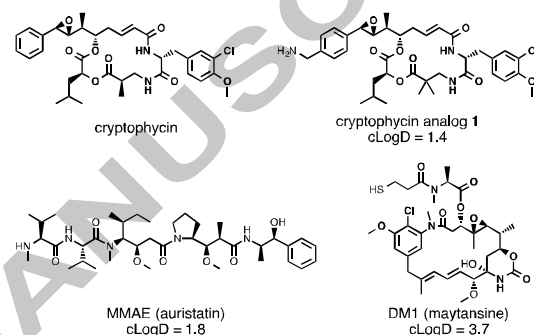


Figure 1. Cryptophycin, cryptophycin analog **1**, MMAE and DM1. cLogD calculated at pH 7.4.

The first step in evaluating the potential for a cryptophycin to function as an effective ADC payload was to confirm its single agent potency. As such, cryptophycin analog **1**²⁰ was assayed against a panel of cell lines comprising a variety of cancer cell types (Table 1). The potent picomolar activity of compound **1** against this panel was consistent with potencies reported in the literature for the cryptophycin class²¹ and indicated its potential to be effective as an ADC agent. Moreover, comparison to the approved ADC payload classes of the auristatins and the maytansines indicated potency increases of one to two orders of magnitude for cryptophycin **1** (Table 1).

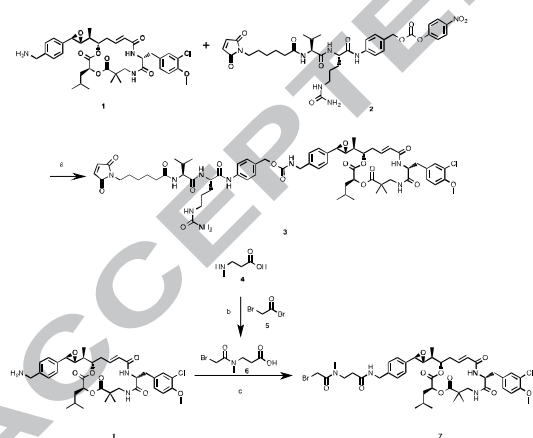
Cell line	IC ₅₀ (nM)		
	1	MMAE	DM1SMe
MDA-MB-231 (breast)	0.044	1.7	0.22
KPL-4 (breast)	0.014	0.23	0.060
MES-SA (uterine)	0.034	1.3	0.18
HCT-116 (colon)	0.024	0.49	0.079
DLD-1 (colon)	0.059	3.7	0.18
A2058 (skin)	0.015	0.44	0.063
BJAB (B-cell)	0.030	0.55	0.11

HL-60 (AML)	0.014	0.30	0.067
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Table 1. *In-vitro* activity of compound **1**, MMAE, and DM1SMe (methylated-DM1).

Two different strategies were envisioned utilizing the cryptophycins: a protease cleavable,^{24,25} releaseable linker that would release free cryptophycin analog **1** in a cell and a non-cleavable linker that would be broken down in the lysosome to a cysteine-containing cryptophycin linker-drug²⁶. These were targeted in order to mimic the mechanisms of drug release of approved ADCs ADCETRIS® (cleavable)^{27,28} and KADCYLA® (non-cleavable).^{29,30} Additionally, the connection functionality to the antibody for one linker-drug was varied from a maleimide, which has the potential to undergo a reverse-Michael reaction leading to loss of linker-drug, to a bromo-acetamide, which does not have the same liabilities.³¹

The synthesis of the dipeptide valine-citrulline cryptophycin linker-drug was accomplished via the coupling of cryptophycin analog **1**²¹ with linker carbonate **2**^{24,32} to give linker-drug **3** (Scheme 1). To synthesize the cryptophycin non-cleavable linker-drug, acid **6**, prepared by acylation of amine **4** with bromoacetyl bromide³³, was coupled with **1** via EEDQ-mediated amide formation to give the bromo-acetamide **7** (Scheme 1).



Scheme 1. Synthesis of cryptophycin-linker targets. (a) HOBT, pyr., DMF, rt, 65%. (b) KOH, H₂O, 0 °C, 17%. (c) EEDQ, MeOH, CH₂Cl₂, rt, 62%.

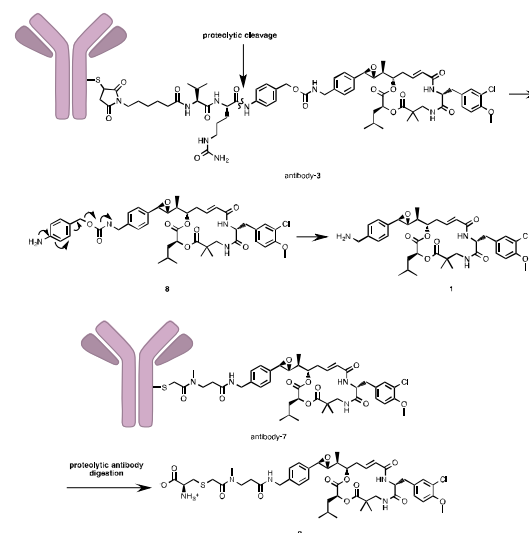
With the cryptophycin linker-drugs **3** and **7** in hand, conjugations were performed utilizing our site-specific, engineered cysteine-based THIOMAB technology³⁴ to generate the TDC (Thiomab-Drug Conjugate) (Table 2). Both cryptophycin-linker

compounds conjugated well, with high drug-to-antibody ratios (DAR) and low aggregation levels, consistent with the hydrophilic nature of the cryptophycins. Both **3** and **7** were conjugated to the cysteine mutant A118C for HER2 and CD22.

Compound	Target	DAR	Aggregation
3	HER2	1.7	3%
	CD22	1.6	2%
7	HER2	2.0	2%
	CD22	2.0	2%

Table 2. Conjugation of compounds **3** and **7**. DAR = drug:antibody ratio.

The active compounds presumed to be released through TDC internalization and enzymatic activity are different for the cleavable and non-cleavable conjugates (Scheme 2). For antibody-**3**, proteolytic activity in the lysosome²⁴ releases a para-amino benzyl alcohol carbamate, which breaks down through a 1,6-elimination and the release of CO₂ to give free **1**.³⁵ On the other hand, antibody-**7** does not contain an optimized protease cleavable bond in the linker and thus relies on lysosomal degradation of the antibody to its constituent amino acids. Thus, cysteine linked-cryptophycin **9** is the presumptive active metabolite produced from conjugates employing **7**.²⁶



Scheme 2. Breakdown of the cryptophycin TDCs and presumptive active species generated.

The TDCs were evaluated *in vitro* against several cell lines that express the

target antigen. The IC₅₀ of the HER2-3 TDC was evaluated alongside the corresponding HER2 TDC containing the same linker but attached to the auristatin MMAE **8** (ADCETRIS® linker-drug), HER2-**8**. As can be seen in Figure 2, in both HER2 amplified cell lines SK-BR-3 and KPL-4, the cryptophycin TDC was highly potent and more active than the MMAE-containing TDC HER2-**8**. Non-specific activity was minimal, as the corresponding control CD22 conjugate (CD22-**3**) was significantly less active. Moreover, no activity was noted in MCF-7 cell line, which does not express elevated levels of Her2, thus in line with a HER2-targeted process.

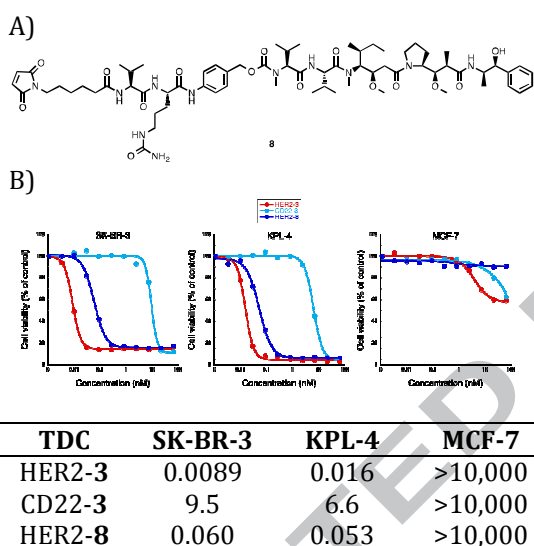


Figure 2. A) Structure of MMAE-linker. B) IC₅₀ curves and values for cryptophycin and MMAE conjugates (nM).

The TDC derived from the bromoacetamide **7** was also tested *in vitro* (Figure 3). As with the TDC generated from releasable linker-drug **3**, this conjugate displayed similar potent activity against HER2 amplified cells while the control CD22-**7** was much less potent.

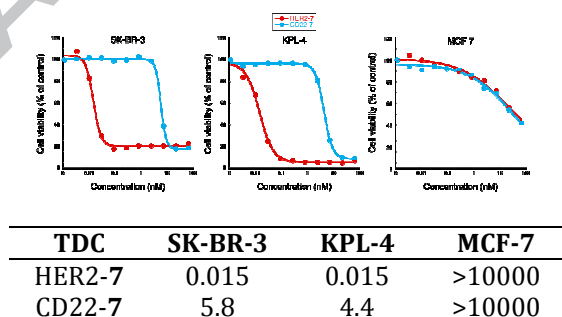


Figure 3. IC₅₀ curves and values for conjugates generated from bromoacetamide **7** (nM).

In addition to evaluating the cryptophycin conjugates against HER2 amplified breast cancer lines, the CD22 cryptophycin conjugate-**3** was tested on B cell lines expressing the target antigen (Figure 4). As with the HER2 TDCs, the CD22 conjugate was highly potent and again more active than the MMAE conjugate (CD22-**8**) by up to an order of magnitude. The HER2 TDC was used as a control and showed a loss of potency, indicating a specific CD22-mediated mechanism of action.

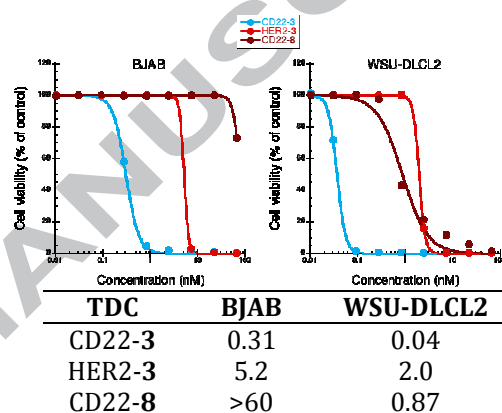


Figure 4. IC₅₀ curves and values for CD22 conjugates generated from cryptophycin and MMAE-linkers (nM).

The cryptophycin analog **1** was evaluated as an ADC payload. The free drug *in vitro* activity was confirmed in a panel of cell lines to be extremely potent. Free payload potency was greater by one to two orders of magnitude compared to auristatin or maytansine derivatives which are approved ADC payloads. Cryptophycin-linkers were successfully synthesized and conjugated to antibodies targeting solid tumors and hematological malignancies, generating TDCs of both the releaseable and non-releaseable variety. These conjugates displayed low levels of aggregation, a common obstacle for more lipophilic ADC toxins, highlighting a physiochemical advantage of the cryptophycin class. *In vitro* activity of these TDCs against both HER2 amplified and CD22 expressing cell lines demonstrated activity, surpassing the potency of the cytotoxic drug MMAE linked to the same antibodies. On target mechanism of action was confirmed both through the use of a control TDC as well

as through the use of cell lines not expressing the target antigen. Further evaluation of the cryptophycins as an exciting class of ADC payloads is ongoing.

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cryptophycin antibody drug conjugate