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Synthesis and Biological Characterization of Monomeric and Tetrameric RGD-Cryptophycin Conjugates

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Abstract: The effective delivery of cytotoxic agents to the tumor cells is a key challenge in anticancer therapy. Multivalent integrin specific ligands are considered a promising tool to increase the binding affinity, selectivity, and internalization efficiency of small molecule-drug conjugates. Here we report the synthesis and biological evaluation of a multimeric conjugate containing the high-affinity integrin $\alpha_v\beta_3$ binding ligand RAFT-c(RGDfK)₄, a lysosomally cleavable Val-Cit linker and cryptophycin-55 glycinate, a potent inhibitor of tubulin polymerization. In vitro cytotoxicity assays verified that the multimeric RGD-cryptophycin conjugate displays improved potency compared to the monomeric analogue in integrin $\alpha_v\beta_3$ overexpressing tumor cell lines, while significantly reduced activity was observed in the antigen-negative cell line.

The selective delivery of anticancer agents to tumor cells constitutes a promising strategy for an optimized therapeutic index and increased clinical benefit in the treatment of cancer. Among these approaches, antibody-drug conjugates (ADCs) employ antibodies that specifically bind to target antigens overexpressed on cancer cells and, thus, confer tumor-specificity to highly potent cytotoxic agents.^[1] Currently six ADCs (Adcetris™, Kadcyla™, Mylotarg™, Besponsa™, Polivy™ and Lumoxiti™) have been approved for oncological indications, while numerous compounds are in different stages of the clinical development.^[2,3] In contrast to ADCs, small molecule-drug conjugates (SMDCs) are considered to have great potential for improved tissue penetration and accelerated tumor accumulation, while not being immunogenic and are obtainable by chemical synthesis.^[4,5]

The heterodimeric transmembrane glycoprotein integrin $\alpha_v\beta_3$ has been a widely exploited target due to its high expression in new tumor blood vessels but also in many cancer types (such as glioblastoma, melanoma, lung, breast, prostate, and ovarian cancer), where it plays a key role in many steps of disease progression and metastasis.^[6,7] A variety of cyclic peptides and peptidomimetics containing the minimum integrin binding motif Arg-Gly-Asp (RGD) have been investigated as high affine and selective $\alpha_v\beta_3$ integrin ligands.^[8,9] Many of them have been used as carriers for the tumor selective delivery of cytotoxic payloads and imaging agents.^[10-12]

Significant advances to further increase the selectivity and binding affinity of the RGD ligands towards integrin $\alpha_v\beta_3$ have been achieved using multivalent systems^[13-16] or by increasing the size of monomeric RGD peptides.^[17] In this context, a multimeric system comprising a regioselectively addressable functionalized template (RAFT) cyclodecapeptide scaffold and four copies of the functionalized cyclopentapeptide c(RGDfK), [RAFT-c(RGDfK)₄], specific for integrin $\alpha_v\beta_3$, is a promising synthetic vehicle for drug delivery and imaging applications.^[18] It was shown that the labeled

tetrameric compound RAFT-c(RGDfK)₄-Cy5 displays a 10-fold higher binding affinity towards isolated integrin $\alpha_v\beta_3$ compared to the monomeric analogue. Additionally, the multimeric ligand efficiently internalizes with the $\alpha_v\beta_3$ receptor, through the clathrin-mediated endocytic pathway.^[19] For this reason, the RAFT-c(RGDfK)₄ demonstrates improved and more specific integrin $\alpha_v\beta_3$ -targeting and imaging properties for in vitro applications, as well as for the in vivo detection and treatment of solid tumors, compared to the monomeric c(RGDfK) peptide.^[14,20–22]

Previously, RAFT-c(RGDfK)₄ was conjugated to a Bax proapoptotic protein derived peptide across a disulfide bridge (RAFT-c[RGD]4-S-S-depsi-cgg-Poro2D). This conjugate displayed a dose-dependent toxicity against Me275 and Colo829 human melanoma cell lines and induced tumor growth inhibition in Me275 xenografts.^[23] However, the RAFT poropeptide conjugate showed a biological activity in the micromolar range, and, therefore, high amounts of the compound were necessary for the treatment. In order to reduce the dosing and increase the efficacy, the application of more active agents was envisioned.

In recent years, considerable research efforts have been devoted to the development of SMDCs based on cryptophycins, a family of microtubule targeting agents, that are characterized with outstanding potency and retained activity against multidrug-resistant (MDR) cancer cell lines.^[24–28] Remarkably, the synthetic cryptophycin-55 glycinate (**1**, Figure 1) displays adequate stability, exhibits cytotoxic activity in the subnanomolar range and shows high antitumor activity in vivo against MDR tumors.^[26,29]

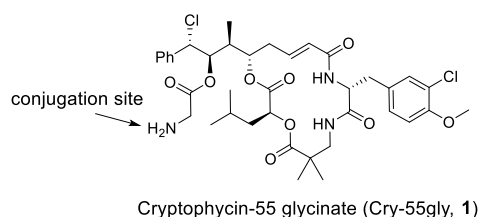
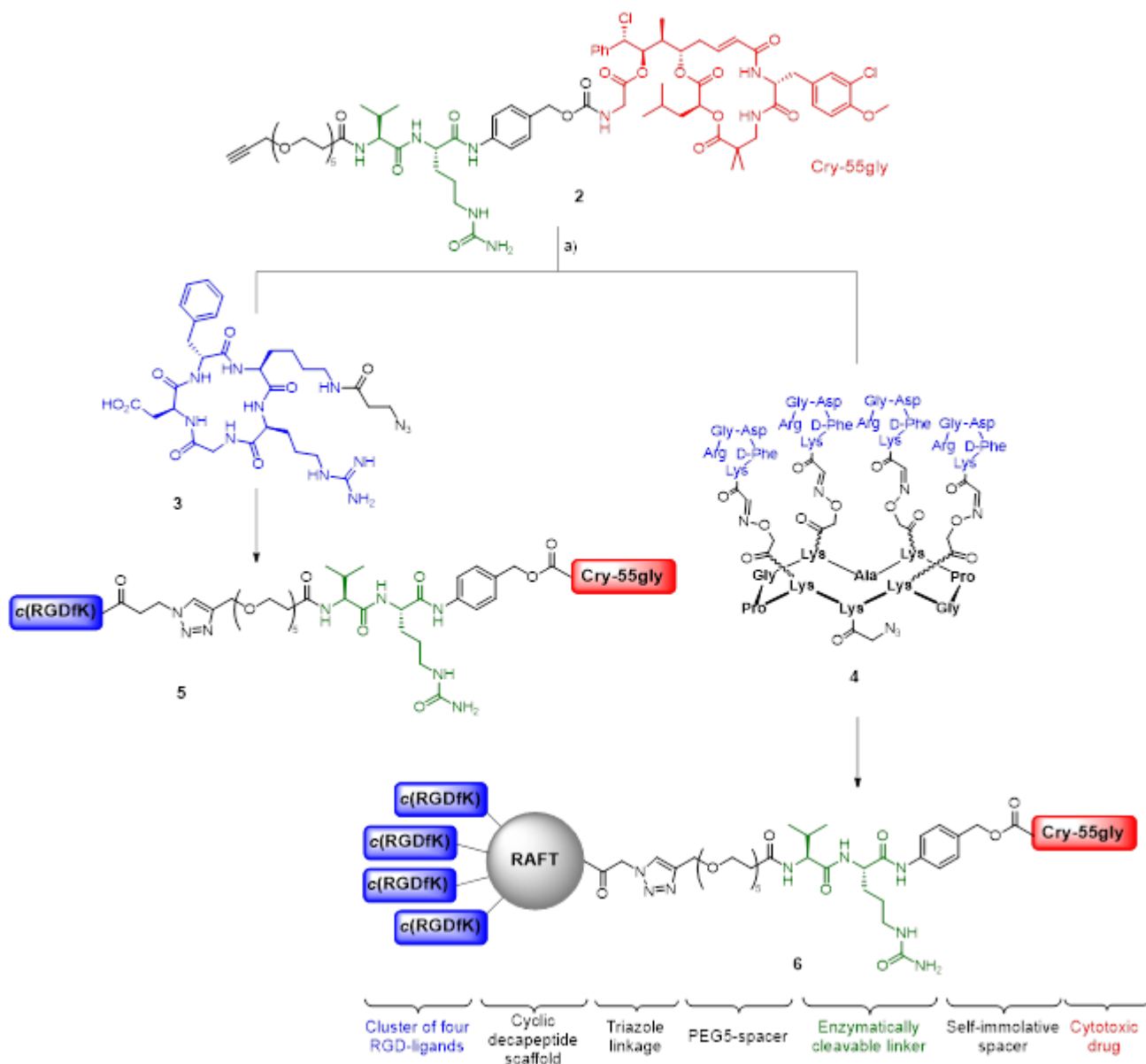


Figure 1. Molecular structure of cryptophycin-55 glycinate

We have previously reported that conjugates of monomeric c(RGDfK) ligands and cryptophycin-55 glycinate display high potency against the M21 and M21-L human melanoma cells.^[26] However, we aimed to improve the tumor targeting properties of RGD-cryptophycin conjugates using multivalent ligands.

Based on previous results, we focus here on the conjugation of the tetrameric RAFT-c(RGDfK)₄ integrin ligand with the highly active cryptophycin derivative, cryptophycin-55 glycinate, aiming at improved selectivity in integrin $\alpha_v\beta_3$ targeted drug delivery. Taking advantage of an efficient intracellular drug release, a cleavable linker was incorporated between the ligand and the cytotoxic agent consisting of a PEG5-chain, the protease sensitive Val-Cit dipeptide, and the para-aminobenzyloxycarbonyl (PABC) self-immolative moiety. Cryptophycin was conjugated to the enzymatically cleavable Val-Cit dipeptide including the PABC moiety via carbamate bond. An alkyne-functionalized PEG5-linker was introduced to the N terminus of the linker to allow the reaction with the azido-functionalized monomeric (**3**) or tetrameric (**4**) integrin ligands (Scheme 1). The conjugate **5** containing the monomer RGD ligand was synthesized as previously reported,^[26] whereas multimeric RGD compound **4** was achieved using a modular convergent strategy that involves the oxime ligation of aldehyde-RGD and RAFT that displays 4 aminoxy groups (see Supporting Information).^[18] The multimeric conjugate **6** was obtained by the copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) between the azido-functionalized RAFT-c(RGDfK)₄ ligand **4** and the alkyne-functionalized linker-cryptophycin intermediate **2**. The final conjugate was purified by preparative HPLC and characterized by analytical HPLC and HRMS (See Supporting Information).



Scheme 1. Synthesis of conjugates **5** and **6**. Reagents and conditions: a) **3** or **4**, CuSO₄·5H₂O, sodium ascorbate, 1:1 DMF/H₂O, 40 °C, 24 h.

The antiproliferative activity of the conjugates was evaluated using three cell lines expressing different levels of integrin $\alpha_v\beta_3$. The U87 human glioblastoma and M21 human melanoma cells were selected based on their high expression of integrin $\alpha_v\beta_3$, while the M21-L human melanoma cell line, a stable variant of M21 that specifically lacks the α_v subunit, was used as negative control.^[30–32] In a first set of experiment, cells were incubated with increasing concentrations from 0.1 to 10 nM of the free drug, RAFT-c(RGDfK)⁴ or conjugates **5** and **6** for 72 hours and cell viability was determined by MTS assay (Figure 2, Figure S1). The calculated IC₅₀ values are shown in Table 1.

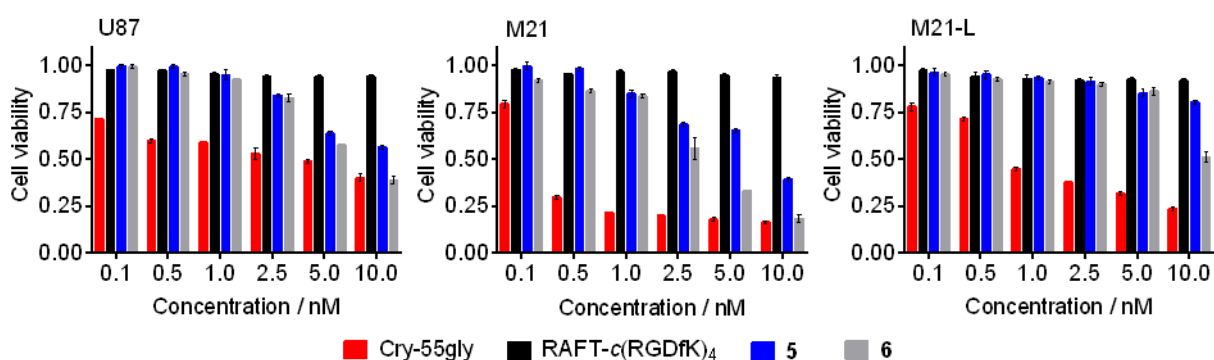


Figure 2. In vitro cytotoxicity of cryptophycin-55 glycinate, RAFT-c(RGDfK)₄, monomeric (**5**) and tetrameric (**6**) RGD-cryptophycin conjugates in U87 human glioblastoma, M21 and M21-L human melanoma cells upon 72 h treatment. Data are represented as mean ± SD (n = 3).

Table 1. Cytotoxic potencies of free cryptophycin-55 glycinate, RAFT-c(RGDfK)₄, monomeric (**5**) and tetrameric (**6**) conjugates against U87 human glioblastoma, M21 and M21-L human melanoma cell lines upon 72 h treatment.

Compound	IC ₅₀ / nM	IC ₅₀ / nM	IC ₅₀ / nM
	U87 (α _v β ₃ +)	M21 (α _v β ₃ +)	M21-L (α _v -, α _v β ₃ -)
Cry-55gly	1.64	0.28	0.86
RAFT-c(RGDfK) ₄	> 10	> 10	> 10
RGD-Cry-55gly (5)	> 10	7.65	> 10
RGD ₄ -Cry-55gly (6)	6.65	2.53	> 10

As expected, the unconjugated RAFT-c(RGDfK)₄ ligand had no or minimal antiproliferative effects, while cryptophycin-55 glycinate was highly active and induced a significant cell growth inhibition (Figure 2). After exposition to a 10 nM concentration of drug, more than 50% cell death was observed in case of U87 cells and more than 75% for M21 and M21-L cells. The low nanomolar and subnanomolar IC₅₀ values of the unconjugated drug underline its high potency (Table 1). Nevertheless, the U87 showed a 6-fold, while the M21-L cell line displayed a 3-fold decreased sensitivity to cryptophycin than the M21 cell line.

The integrin positive cells U87 and M21 displayed a dose-dependent inhibition of cell growth upon treatment with the tetrameric and monomeric RGD-cryptophycin conjugates, both compounds exhibiting IC₅₀ values in the nanomolar range. In strong contrast, incubation of the M21-L cells with conjugates **5** and **6** resulted in marginal cell growth inhibition, similar to that observed for the unconjugated ligand.

In U87 cells, only a minimal difference was found between the activity of both conjugates, the conjugate **6** containing the tetrameric ligand being slightly more active at each tested concentration. At the same time, the activity of multivalent conjugate **6** was 3-fold higher compared to the monomeric

conjugate **5** (IC_{50} = 2.53 nM and 7.65 nM, respectively) when tested in M21 melanoma cells. Remarkably, the multimeric conjugate **6** showed the same toxicity as the free cryptophycin-55 glycinate at the highest concentration (10 nM), while monomeric conjugate **5** demonstrated a reduced activity. This clearly underlines the improved internalization and integrin $\alpha_v\beta_3$ -targeting properties of the multimeric structure and ensures a greater tumor selectivity. Moreover, significantly reduced activity of conjugates was observed in M21-L cells ensuring greater tumor selectivity, but also signifying stability of the conjugates **5** and **6** in cell media.

In a second set of experiments, M21 and M21-L cells were incubated with increasing doses from 1 to 25 nM of the free cryptophycin-55 glycinate or conjugates **5** and **6** for 72 hours and cell viability was analyzed by MTS assay (See Supporting information Figure S2, Table S1). In parallel with above presented findings, the multimeric conjugate **6** showed approximately 3-fold increased activity compared to the monomeric conjugate **5**, while the potency of both conjugates was greater on the integrin positive M21 cell line.

Finally, conjugation of the tetravalent RGD-ligand to the antimitotic agent cryptophycin across intracellularly cleavable linker, has dramatically improved the potency of targeted SMDC based on this ligand, compared to the previously reported RAFT-c[RGD]₄-S-S-depsi-cgg-Poro2D conjugate.^[23] These results underscore the importance of using highly active cytotoxic agents in the context of targeted therapy and show promise for future application of this payload and its derivatives.

Altogether, these results suggest that the RGD-containing scaffold is highly effective for the delivery of potent anticancer agents, such as cryptophycin. The tetrameric RGD-cryptophycin conjugate displays impressive potency in vitro in different cell lines expressing $\alpha_v\beta_3$ integrin, especially in M21 melanoma cells. On the basis of the previous and current results, we were able to confirm that the multimeric RAFT-c(RGDfK)₄ enhances the selectivity of c(RGDfK) and improves tumor targeted drug delivery, providing a rationale for its future therapeutic applications in combination with cytotoxic agents.

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