Drug Delivery

Linker Hydrophilicity Modulates the Anticancer Activity of RGD–Cryptophycin Conjugates

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Abstract: Most anticancer agents are hydrophobic and can easily penetrate the tumor cell membrane by passive diffusion. This may impede the development of highly effective and tumor-selective treatment options. A hydrophilic β-glucuronidase-cleavable linker was used to connect the highly potent antimitotic agent cryptophycin-55 glycinate with the $\alpha_v\beta_3$ integrin ligand *c*(RGDfK). Incorporation of the self-immolative linker containing glucuronic acid results in lower cytotoxicity than that of the free payload, suggesting that hydrophilic sugar linkers can preclude passive cellular uptake. In vitro drug-release studies and cytotoxicity assays demonstrated the potential of this small molecule–drug conjugate, providing guidance for the development of therapeutics containing hydrophobic anticancer drugs.

Introduction

Many commonly used anticancer drugs do not preferentially accumulate at the tumor site, which leads to systemic side effects and to suboptimal therapeutic efficacy.[1] This limitation can be overcome by a ligand-targeted drug delivery approach.[2] Covalent conjugation of cytotoxic agents to antibodies, peptides, or small molecule ligands, capable of selective binding to receptors abundant on the tumor cell surface, enables drug accumulation at the tumor site while decreasing off-target toxicity.[3,4] In this context, nine antibody-drug conjugates (ADCs) have received marketing approval in cancer therapy so far, while more than 65 are currently under clinical investigation.[5,6] Although ADCs have shown therapeutic benefits in clinical trials, they also display significant drawbacks, such as limited intratumor penetration, high manufacturing costs and potential immunogenicity.[7] On the other hand, small molecule–drug conjugates (SMDCs) have attracted considerable interest as a valid alternative to ADCs due to their advantageous pharmacokinetic profile, simpler and more affordable synthetic routes and lack of immunogenicity.[8] Their smaller size enables rapid and homogeneous diffusion into tissue, potentially resulting in high tumor/organ ratio.[9] Like ADCs, SMDCs are composed of a cytotoxic agent and a targeting ligand (homing device) covalently assembled across a linker, which provides sufficient stability during circulation and allows efficient drug release at the site of the disease.[10,11] Such constructs must be designed to safeguard cellular uptake of the payload for example, by receptor-mediated endocytosis,[2] or to liberate the cytotoxic drug extracellularly in the tumor microenvironment.[12,13]

Among the tumor-associated receptors, the heterodimeric transmembrane glycoprotein integrin $\alpha_v\beta_3$ is considered a potential tumor target due to its overexpression on cancer cell surfaces and blood vessels of several solid tumors (e.g., breast cancer, glioblastoma, pancreatic tumor, prostate carcinoma).[14,15] The α_v integrin subtype plays a central role in many stages of cancer progression such as angiogenesis, tumor growth, apoptosis resistance, and metastasis.[16] Integrin $\alpha_v\beta_3$ recognizes and binds the extracellular matrix (ECM) proteins through the minimal tripeptide sequence Arg-Gly-Asp (RGD).[17,18] Consequently, several cyclic RGD-bearing peptides and peptidomimetics have been prepared and conjugated to different cytotoxic agents.[19] Specifically, the cyclopentapeptide *c*(RGDfK) has been widely exploited as targeting ligand for imaging,[20] diagnostic[21] and drug delivery applications,[22] due to its nanomolar binding affinity and the Lys conjugation handle.[23,24]

Besides to cytotoxic agents commonly used in chemotherapy regimens,[1] the cryptophycins have also been recently considered as drug candidates for targeted tumor therapy.[25] Cryptophycins are natural occurring 16-membered macrocyclic depsipeptides produced by cyanobacteria.[26] This class of compounds exhibits potent cytotoxicity toward several cancer cells

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including multidrug-resistant (MDR) cells.[27] Their strong antiproliferative activity is based on the irreversible inhibition of the β-tubulin polymerization during mitosis, leading to cell-cycle arrest in G_2/M phase and activation of apoptosis pathways.[28] Although the expected clinical success could not be achieved using cryptophycin-52 (**4**) as stand-alone agent,[29,30] these compounds have emerged as potent payloads in the targeted therapy approach. For example, different cryptophycin-based ADCs developed by Sanofi and Genentech have shown promising preclinical results.[31–33]

Moreover, we have contributed to this field with the development of tumor targeting ADCs[34] and SMDCs[35–39] bearing the potent cryptophycin-55 glycinate (**3**) as anticancer payload. This cryptophycin derivative possesses distinct features, such as high in vitro potency, remarkable in vivo activity against MDR xenografts,[40] excellent stability in mouse and human plasma[36] making it suitable for active tumor targeting.

We recently reported the development of first-generation RGD–cryptophycin conjugates[36] containing the enzymatically cleavable Val-Cit linker (**X1** and **X2**, see Supporting Information Figure S4) and found that conjugates display high in vitro potency but poor selectivity toward M21 and M21-L human melanoma cell lines with different $\alpha_v\beta_3$ integrin expression levels. We proposed that the nonspecific passive cellular uptake of the conjugates could be associated with the high payload hydrophobicity.[36] Nevertheless, drug-linkers with improved hydrophilicity provide optimal pharmacokinetic properties to the overall construct that may prevent aggregation and/or passive permeation across the cell membrane.[41,42] To this end, the protease-sensitive β-glucuronide can be incorporated into the linker system as a hydrophilic alternative to Val-Cit linkers, to minimize the hydrophobicity and permit an efficient drug release.[43–45] The β-glucuronidase is responsible for the hydrolysis of glucuronyl–O bonds and it can selectively activate glucuronide prodrugs accumulated at antigen-positive cancer cells.[46,47]

Results and Discussion

Design

Herein we report the first β-glucuronidase-cleavable conjugate equipped with the potent antimitotic agent cryptophycin-55 glycinate (Cry-55gly) and the $\alpha_v\beta_3$ integrin ligand *c*(RGDfK), suitable for the targeted therapy of solid tumors. This drug delivery system was designed to be selectively activated by the tumor-associated enzyme β-glucuronidase present at high concentrations intracellularly in lysosomes and in necrotic tumor environment of many malignancies including lung, breast, ovarian, gastrointestinal tract carcinomas, and melanomas.[46,47] Upon enrichment and binding to integrin $\alpha_v\beta_3$, the active cytotoxic drug can be liberated from the RGD–cryptophycin conjugate both inside the target cells[48] and in the extracellular tumor environment, from where it can diffuse into surrounding cancer cells.[49]

The central self-immolative linker covalently connects the main components of the system, ensuring an efficient drug release after the enzymatic cleavage of the β-glucuronide trigger located at the *para* position on the aromatic core (**1**, Figure 1). The synthesis of a negative control to monitor the drug-release efficiency was envisaged by positioning the β-glucuronide (β-GlcA) in *meta* position on the aromatic ring of the linker (**2**, Figure 1). In the latter case the enzymatic cleavage is not followed by self-immolation step, thus a conjugate-intermediate with decreased activity is expected.

Synthesis

Jeffrey et al., reported the synthesis of conjugates between β-glucuronide linkers and monomethyl auristatin E (MMAE), by reacting an acetyl and methyl ester protected GlcA derivative with the cytotoxic agent. The protecting groups of the sugar moiety were removed by treatment with LiOH to provide the

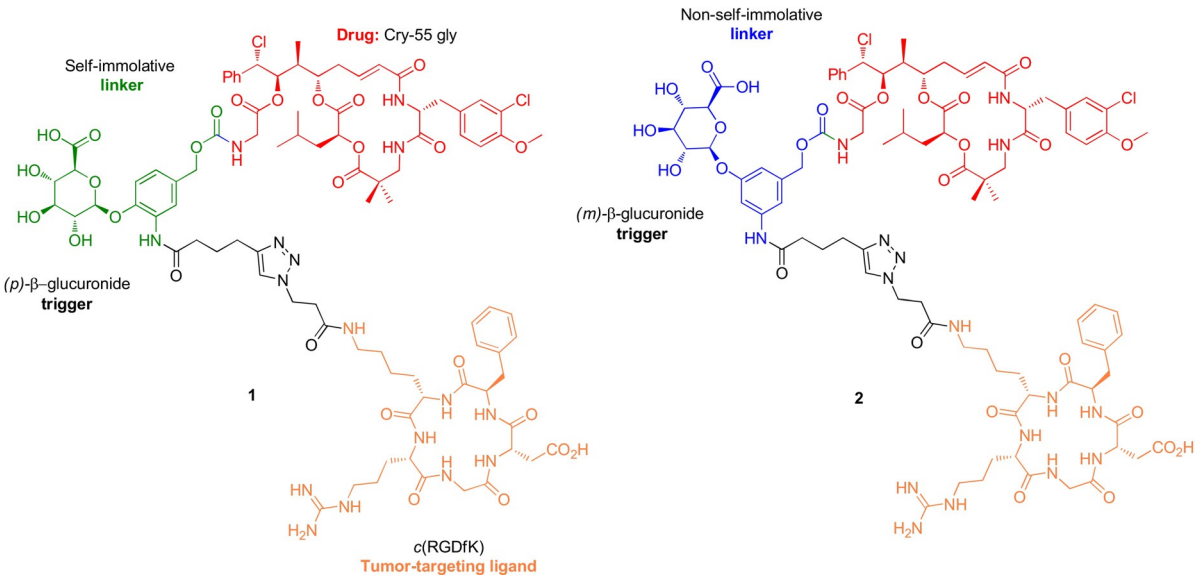

Figure 1. Structures of self-immolative conjugate *c*(RGDfK)-(*p*)-GlcA-linker-Cry-55gly **1**, and non-self-immolative conjugate *c*(RGDfK)-(*m*)-GlcA-linker-Cry-55gly **2**.

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drug-linker intermediate ready to be conjugated to the respective tumor targeting ligand.[43] However, this synthetic strategy was expected unfeasible with Cry-55gly, due to its instability under alkaline conditions. In a pilot experiment, treatment of Cry-55gly with $LiOH/H_2O$ at 0 °C for 30 minutes resulted in 70% conversion into the epoxide containing cryptophycin derivative (cryptophycin-52, **4**) as determined by HPLC–MS (Scheme 1A, see Supporting Information). This observation is consistent with the conversion of other cryptophycin analogues into the corresponding epoxides under various conditions (i.e., chlorohydrins are transformed into epoxides at physiological pH or in aqueous solution).[40]

An alternative synthetic route was developed to avoid payload decomposition during synthesis, involving the exchange of the protecting groups on the carbohydrate moiety (Scheme 1B). Conjugate **1** was prepared from commercially available 4-hydroxy-3-nitrobenzaldehyde (**5**). Stereoselective glycosylation of **5** to acetobromo-α-D-glucuronic acid methylester (**6**) was performed under Koenigs–Knorr conditions in the presence of silver oxide as catalyst affording **7** in 93% yield. This was followed by aldehyde reduction with sodium borohydride providing **8** in 95% yield without the need of purification. The corresponding benzylic alcohol was treated with *tert*-butyldimethylsilyl chloride and imidazole to produce the silyl ether protected derivative **9** (93%). At this stage, as reported by Grinda et al., the protecting groups of the β-glucuronide were modified via a three-step strategy to yield the fully allyl-protected carbohydrate (**12**).[50] This methodology offers a stable and compatible glucuronide protection in the course of the synthesis, while the deprotection can be performed in a one-step procedure under mild conditions at the end of the synthesis. The acetyl groups were removed from **9** using sodium methoxide to afford the hydroxy-free derivative **10** (84%). Transesterification of the methyl ester with sodium allylate gave the allyl ester **11** in 83% yield. The three allyl carbonates were introduced in the presence of a large excess of allyl chloroformate using pyridine as solvent. After three days, the fully allyl protected glucuronide **12** was obtained in 82% yield. Subsequently, nitro reduction with zinc powder under acidic conditions gave the free aniline (**13**, 88%) which was subsequently coupled with 5-hexynoic acid in the presence of EEDQ providing **14** (92%) with a suitable alkyne-functionalized spacer. Removal of *tert*-butyldimethylsilyl group was carried out with HF/pyridine to yield the free benzyl alcohol **15** (93%) which was subsequently treated with 4-nitrophenyl chloroformate and pyridine to give the activated carbonate **16** in quantitative yield. Cry-55gly[35] (**3**) was introduced via nucleophilic substitution in the presence of DIPEA to afford the carbamate **17** in 77% yield after RP-HPLC purification. Full allyl deprotection of the glucuronide moiety was carried out using catalytic amount of tetrakis(triphenylphosphine)palladium(0) affording the Cry-55gly linker intermediate **18** in 66% yield after RP-HPLC purification. Finally, the conjugation to the targeting ligand *c*(RGDfK) **33**, properly modified with 3-azidopropanoic acid on the Lys side chain (**32**, see Supporting Information), was achieved by triazole formation. The copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) was carried out at 35 °C for 24 h in the presence of alkyne **18** and the azido-cyclopeptide derivative **33**, using $CuSO_4$ and sodium ascorbate. After purification by preparative RP-HPLC, the final conjugate **1** was obtained in 84% yield.

Following a similar synthetic approach, the conjugate **2** equipped with the β-glucuronide moiety in *meta* position on the linker was also prepared (negative control). Firstly, the precursor 3-(hydroxymethyl)-5-nitrophenol (**20**) was synthesized by reduction of the commercially available 3-hydroxy-5-nitrobenzoic acid **19** using a solution of 1.0 M BH_3·THF in THF at 0 °C (94%). The resulting benzylic alcohol of **20** was selectively protected with *tert*-butyldimethylsilyl chloride in the presence of imidazole at 0 °C producing the protected silyl ether derivative **21** (61%). Then, the free hydroxy group in *meta*-position was coupled with acetobromo-α-D-glucuronic acid methylester **6** under the same Koenigs-Knorr conditions that yielded **7**, to give **22** with 80% yield. By following the same synthetic Scheme described above, the RGD–cryptophycin conjugate **2** was obtained in 43% yield. The final conjugates **1** and **2** were characterized by analytical HPLC and HRMS (see Supporting Information).

Integrin binding affinity

Conjugates **1** and **2** were evaluated for their ability to compete with vitronectin binding to the isolated $\alpha_v\beta_3$ receptor. The binding affinity was assessed using a competitive ELISA-based assay and it was compared with the affinity of the free peptide **33** (Figure 2). Integrin binding assays were carried out by incubation of $\alpha_v\beta_3$ integrin with increasing concentrations of the conjugates (10^{-5}–10^{-12} M) in presence of the ECM immobilized protein vitronectin. Peptide **33** showed an IC_{50} value of 0.81 nM, similar to that of the reference cilengitide (IC_{50} = 0.54 nM),[24] confirming that functionalization with 3-azidopropanoic acid did not affect the integrin binding. Conjugates **1** and **2** retained good binding affinity to the receptor with IC_{50} values in the nM range (21.9 nM and 11.7 nM, respectively) indicating that the increased size and the steric bulk of these conjugates cause only a modest decrease in affinity (Figure 2).

β-Glucuronidase-catalyzed release of cryptophycin-55 glycinate

The drug-release mechanism involves the enzymatic hydrolysis of the glycosidic bond from the linker followed by the spontaneous self-immolative process with concomitant loss of carbon dioxide and release of the active drug (Scheme 2).[51]

The drug-release efficiency was tested by treating the conjugates **1** and **2** with *E. coli* β-glucuronidase (200 U mL^{-1}) at 37 °C. The release of Cry-55gly (**3**) was monitored over a period of 60 min by analytical HPLC, followed by analyte identification using UPLC–MS. As expected, the conjugates **1** and **2** were rapidly cleaved upon incubation with the enzyme (Figure 3). In detail, the enzymatic cleavage of **1**, with *para*-substituted β-glucuronide moiety, generated the metabolite **M1** that rapidly underwent 1,6-elimination releasing the active Cry-55gly payload (Figure 3A, full characterization and MS spectra are in

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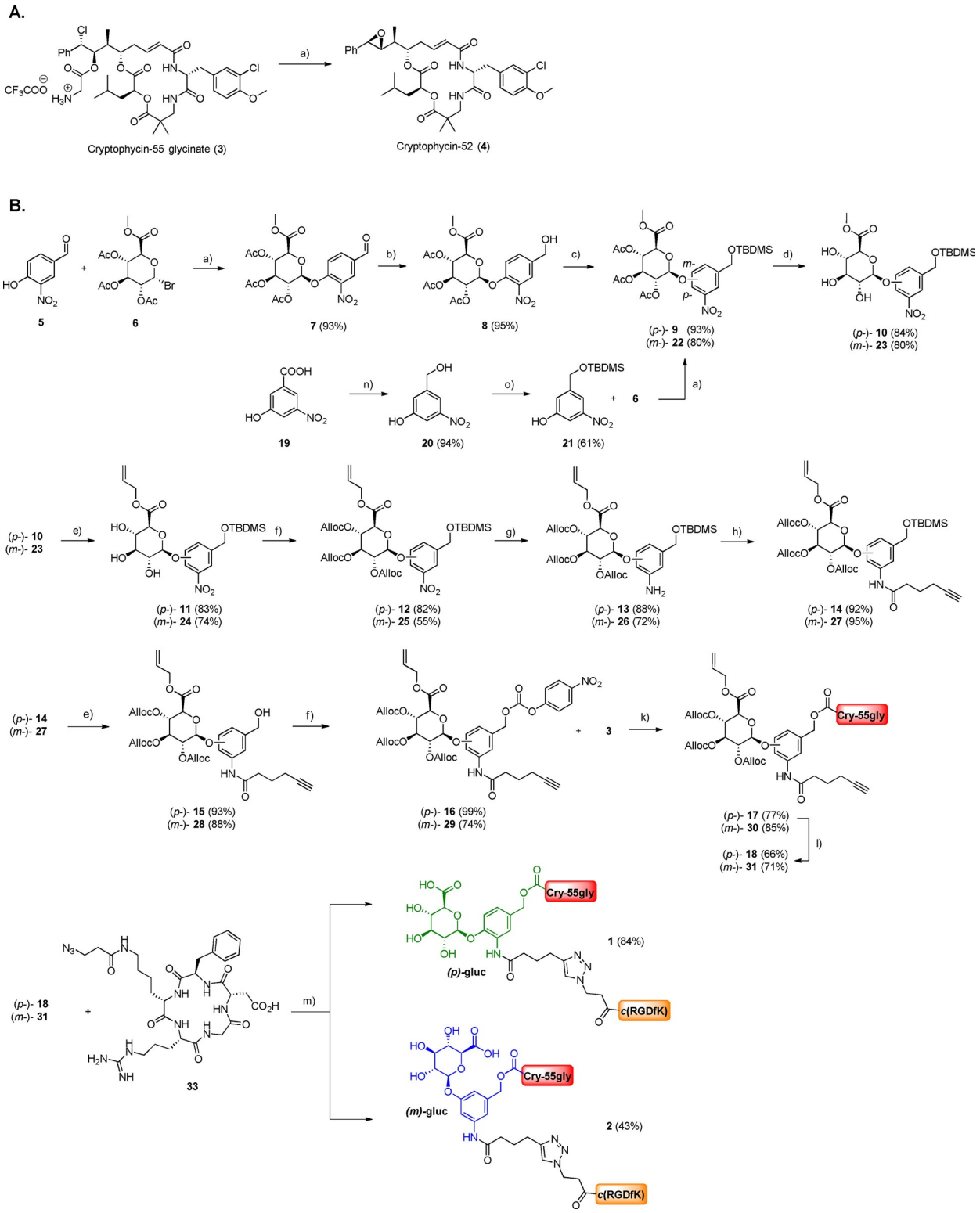

Scheme 1. A) Epoxide formation side reaction: a) LiOH, H_2O, 30 min, 0 °C, 70% conversion. B) Synthesis of conjugates **1** and **2**: a) Ag_2O, CH_3CN, darkness, RT, 4 h; b) $NaBH_4$, $CHCl_3$/*i*PrOH (5:1), silica gel, 0 °C, 45 min; c) imidazole, TBDMSCl, CH_2Cl_2, RT, 12 h; d) MeONa 30% *w*/*v*, MeOH, 0 °C, 1.5 h; e) sodium allylate 0.126 M, allylic alcohol, RT, 40 min; f) allyl chloroformate, pyridine, RT, 72 h; g) zinc, MeOH/AcOH (10:1), RT, 30 min; h) 5-hexynoic acid, EEDQ, CH_2Cl_2, RT, 24 h; i) HF/pyridine 70%, THF, RT, 1 h; j) 4-nitrophenyl chloroformate, pyridine, CH_2Cl_2, 0 °C→RT, 2 h; k) DIPEA, DMF, RT, 4 h; l) $Pd(PPh_3)_4$, morpholine, CH_2Cl_2, RT, 1 h; m) $CuSO_4$·5 H_2O, sodium ascorbate, DMF/H_2O (1:1), 35 °C, 24 h; n) BH_3·THF, THF, 0 °C→RT, overnight; o) imidazole, TBDMSCl, THF, 0 °C, 2 h.

Supporting Information, Figure S3). In contrast, the β-glucuronidase mediated linker cleavage of the *meta*-substituted conjugate **2** led to the rapid formation of metabolite **M2**, but this was not followed by 1,6-elimination (self-immolative step) and the Cry-55gly was not released over time (Figure 3B). Control experiments indicated that both conjugates were stable in the

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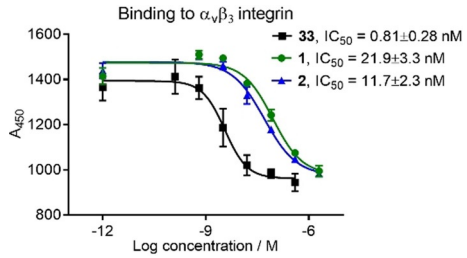


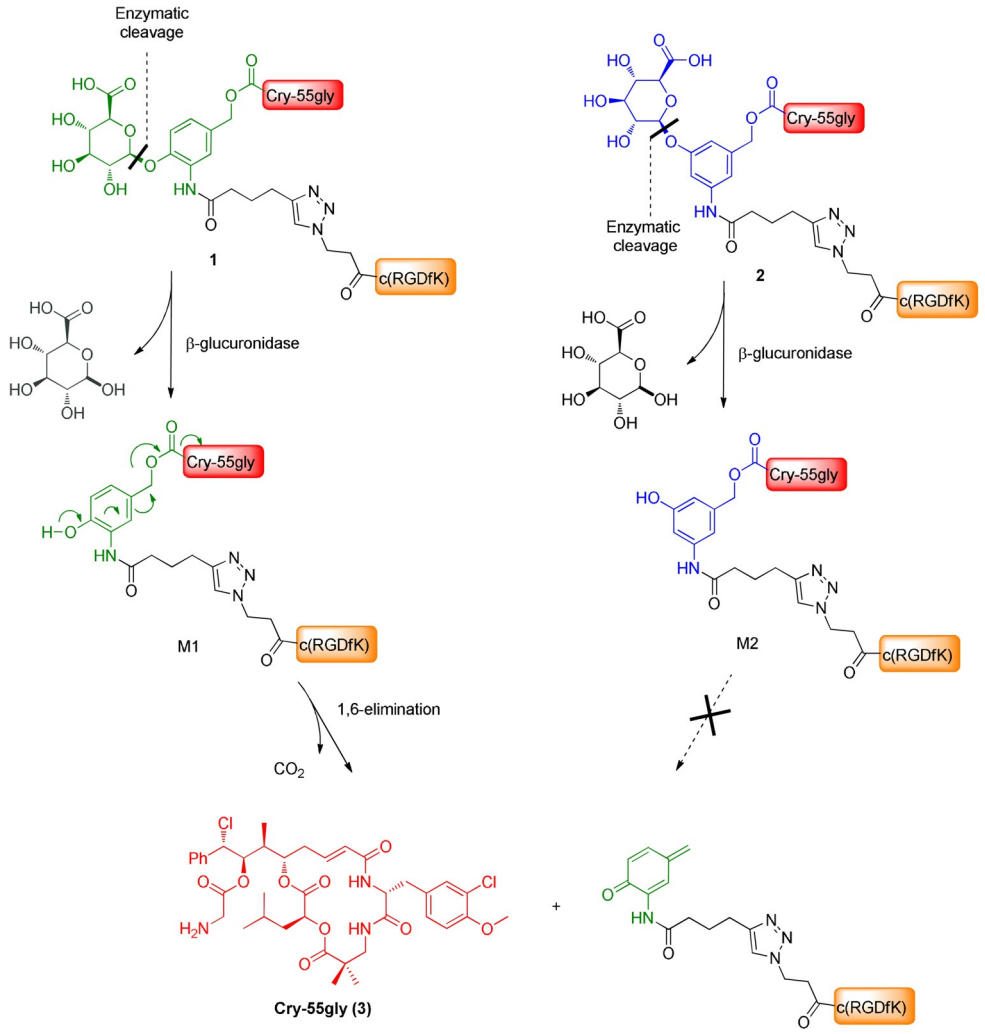
Figure 2. Affinities of conjugates **1**, **2** and reference compound **33** to human integrin $\alpha_v\beta_3$.

absence of enzyme confirming an efficient linker self-immolative process only in the presence of β-glucuronidase.

These results show a rapid ($t_{1/2}$ = 15 min) and virtually complete enzymatic degradation of the conjugate **1** despite the relatively short linkage connecting the β-glucuronidase responsive linker to the cyclic integrin ligand. This is in contrast to recently published results of López Rivas et al., in which inefficient enzymatic cleavage and the lack of regained in vitro activity in the presence of β-glucuronidase were attributed to a suboptimal distance between the enzymatic cleavage site and the ligand in a *cyclo*[DKP-RGD]-GlcA-MMAE conjugate.[49] Albeit the close similarity in terms of distance, our system contains a 1,4-triazole unit that can induce a conformational turn and provide better accessibility to β-glucuronidase.[52] In a close comparison between our conjugate and the efficient system reported by López Rivas et al. (conjugate bearing a PEG_4 spacer), both contain a 1,4-disubstituted triazole adjacent to the β-glucuronidase-cleavable moiety which points out that a conformational turn may be the key element rather than the distance.

In vitro cytotoxicity assay

The in vitro cytotoxic activity of the RGD–cryptophycin conjugates was tested against the $\alpha_v\beta_3$ integrin expressing M21 human melanoma cells.[53,54] Cell viability was measured by resazurin assay after 2 h treatment with increasing concentrations of the free drug and conjugates **1** and **2** in the absence or presence of β-glucuronidase (2 U $well^{-1}$) for 2 h and additional 70 h incubation (Figure 4, Table 1). As the exact β-glucuronidase expression level is unknown in these cancer cells, this model aimed to more closely resemble the tumor microenvir-



Scheme 2. β-Glucuronidase-mediated cleavage, self-immolative mechanism and Cry-55gly release from conjugates **1** and **2**.

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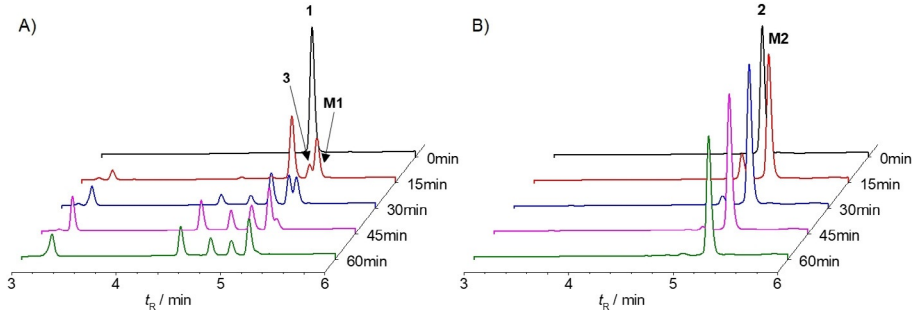

Figure 3. Degradation of conjugates **1** (A) and **2** (B) in the presence of β-glucuronidase. HPLC chromatograms show degradation of **1** upon incubation with *E. coli* β-glucuronidase (200 U mL^{-1}) in PBS at 37 °C within 60 min, as well as the formation of metabolites **M1** and Cry-55gly (**3**), while the degradation of **2** leads to the formation of metabolite **M2**.

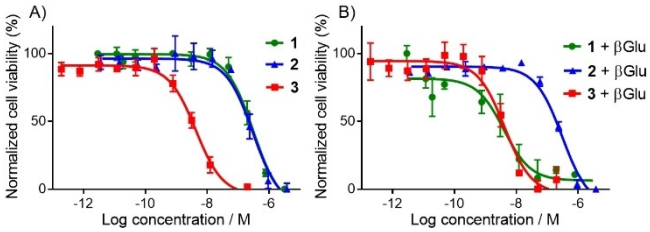

Figure 4. Cytotoxic effect of Cry-55gly (**3**), conjugates **1** and **2** against M21 human melanoma cells in the absence (A) or presence (B) of β-glucuronidase after 2 h treatment and additional 70 h incubation. Curves were obtained by nonlinear regression (four-parameter dose–response); each point represents the mean ± standard deviation of quadruplicates, and the measurements were repeated twice.

Table 1. Cytotoxicity of Cry-55gly (**3**) and conjugates **1** and **2** against M21 human melanoma cells in the absence or presence of β-glucuronidase (βGlu).

Structure	IC_{50} [nM][a]	
	Compd	Compd + βGlu
Cry-55gly (**3**)	4.25 ± 0.43	4.22 ± 0.44
c(RGDfK)-(*p*)-GlcA-Cry-55gly (**1**)	309.6 ± 19.2	3.51 ± 0.55
c(RGDfK)-(*m*)-GlcA-Cry-55gly (**2**)	303.0 ± 26.9	308.4 ± 22.0

[a] Data are the mean ± SD of quadruplicates, and measurements were repeated twice.

onment in vivo, where the extracellular cleavage of the β-glucuronidase-responsive linker allows subsequent internalization of the free cryptophycin by passive diffusion into target cancer cells, while ligand internalization is a possible, but not essential step of the process.

As shown in Figure 4, the untargeted cryptophycin-55glycinate (**3**) was highly toxic against the M21 human melanoma cells with IC_{50} values in the low nanomolar range, and its activity remained unchanged in the presence of β-glucuronidase, as expected. When incubated alone, **1** and **2** displayed a 70-fold decreased activity relative to the free drug **3**. Both conjugates were significantly less potent (IC_{50} = 309.6 and 303.0 nM, respectively) than analogous RGD–cryptophycin conjugates bearing a hydrophobic Val-Cit linker (**X1**: IC_{50} = 7.63 nM and **X2**: IC_{50} = 0.15 nM)[36] using the same cell line, under the same conditions. The presence of a hydrophilic carbohydrate linker influences the activity of the conjugate by preventing passive cellular uptake, presumably observed in the case of **X1** and **X2**.[36] The equal efficacy of **1** and **2** in the absence of β-glucuronidase suggests that the conjugates are most probably insensitive to intracellular β-glucuronidase activity, or that enzyme expression may be low in these cells. The observed trend may be associated with a largely reduced non-integrin-mediated uptake due to the enhanced hydrophilicity of the construct or a modest $\alpha_v\beta_3$-mediated internalization process.

Remarkably, the antiproliferative activity of **1** (IC_{50} = 3.51 nM) in the presence of β-glucuronidase was similar to that of Cry-55gly (IC_{50} = 4.22 nM), demonstrating that extracellular linker activation led to an efficient payload release. This clearly underlines the fast and efficient enzymatic conversion of **1** to the free drug cryptophycin-55glycinate (**3**) (vide supra). In contrast, the cytotoxicity of **2** remained very low also in the presence of β-glucuronidase (IC_{50} = 308.4 nM). This illustrates that the cryptophycin payload is not active when it is not released from the targeting ligand. On the other hand, the metabolite **M2** formed in situ (Scheme 2) displayed modest in vitro antitumor activity, and its transport through the cell membrane differs from that of the free payload.

Conclusions

The development of new cryptophycin-based conjugates bearing spacers with improved hydrophilicity shows a great promise and potential for the targeted therapy of solid tumors.[55] In this work, a β-glucuronidase-responsive linker, a hydrophilic alternative of the widely used Val-Cit linker, has been used to connect the potent antimitotic agent cryptophycin-55 glycinate with the *c*(RGDfK) integrin ligand. A multistep synthetic route was developed to optimally tailor the central sugar-linker moiety, thus, affording a synthetic methodology compatible with the functional groups of cryptophycin-55 glycinate, susceptible to hydrolysis in alkaline reaction conditions. The β-glucuronidase-induced cleavage enabled fast and efficient release of the active payload from conjugate **1** containing a self-immolative linker. The conjugates showed a 70-fold decreased activity relative to the free drug in $\alpha_v\beta_3$ integrin expressing M21 human melanoma cells, suggesting that hydrophilic sugar link-

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ers can preclude passive cellular uptake. Furthermore, conjugate **1** produced similar cytotoxicity as the payload **3** when β-glucuronidase was added to the cell culture medium, underlining that that extracellular linker activation liberates the active drug.

These results indicate that RGD–cryptophycin conjugates bearing β-glucuronide linker have the potential to be therapeutically effective in vivo against integrin $\alpha_v\beta_3$ overexpressing tumors with high β-glucuronidase activity. In this approach, after binding and accumulation of the conjugate at the tumor site, the free drug, that is released by a β-glucuronidase-mediated activation, can penetrate neighboring cancer cells by passive diffusion causing bystander killing.[56] In line with recent advances,[49,57] this methodology could be further applied for the development of therapeutics containing hydrophobic anticancer drugs (e.g., MMAE, maytansinoids) to prevent their passive uptake by healthy cells.

Experimental Section

Procedures for biological assays, supplementary figures, synthetic procedures and characterization details, along with ^{1}H NMR, ^{13}C NMR, HPLC, MS, and HRMS data can be found in the Supporting Information.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: antitumor agents · beta-glucuronidase · drug delivery · integrin · small molecule drug conjugates

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