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COMMUNICATION

β-Glucuronidase Triggers Extracellular MMAE Release from an Integrin-Targeted Conjugate

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A non-internalizing $\alpha_v\beta_3$ integrin ligand was conjugated to the anticancer drug MMAE through a β-glucuronidase-responsive linker. In the presence of β-glucuronidase, only the conjugate bearing a PEG4 spacer inhibited the proliferation of integrin-expressing cancer cells at low nanomolar concentrations, indicating important structural requirements for the efficacy of these therapeutics.

Among the current pharmacological approaches for cancer treatment, cytotoxic agents are still frequently administered to patients with early-stage or advanced disease, as single agents or in combination with radiation, before or after surgery.[1] The covalent conjugation of these drugs to specific ligands (antibodies, peptides or small molecules) capable of selective binding to tumour-overexpressed receptors, has been proposed as a suitable strategy to increase the tumour accumulation of these therapeutics, sparing healthy tissues and reducing dose-limiting toxicities.[2] In particular, the so-called Antibody-Drug Conjugates (ADCs)[3] and Small Molecule-Drug Conjugates (SMDCs)[4] consist of a targeting unit and a cytotoxic payload covalently assembled through a linker, which should be perfectly stable in plasma and be cleaved at the tumour site. It has been originally postulated that, in order to minimize side-toxicity in healthy cells, the payload should be preferentially released within the intracellular compartments (e.g. lysosomes) of the targeted cancer cell, upon receptor-mediated endocytosis of the conjugate. More recently, the observation of the high mutation rates of cancer cells (which may generate different cell populations, showing different levels of antigen expression and drug resistance)[5] and of the role on disease progression played by cancer-associated host cells (e.g. tumour endothelial cells,[6] fibroblasts,[7] infiltrating immune cells[8]) stimulated the hypothesis that the extracellular release of the drug and its diffusion within the tumour mass may increase the treatment efficacy.[9] In line with these observations, new classes of antigens (collagen IV,[10] fibrin,[11] carbonic anhydrase IX,[12] oncofetal fibronectin[13] and tenascin C[14]) have been explored as targets for non-internalizing conjugates and new linkers for the selective extracellular release of the payload have been proposed.

Since 2012, our group has explored the conjugation of different anticancer drugs to the peptidomimetic compound *cyclo*(DKP-RGD)[15] (compound **1** in Figure 1), a low-nanomolar ligand of integrin $\alpha_v\beta_3$.[16] The latter is a heterodimeric transmembrane glycoprotein overexpressed in a variety of cancer types (e.g. melanoma, glioblastoma, renal cell carcinoma, and tumours of lung, ovary, breast, prostate, and colon), where it is involved in disease progression.[17]

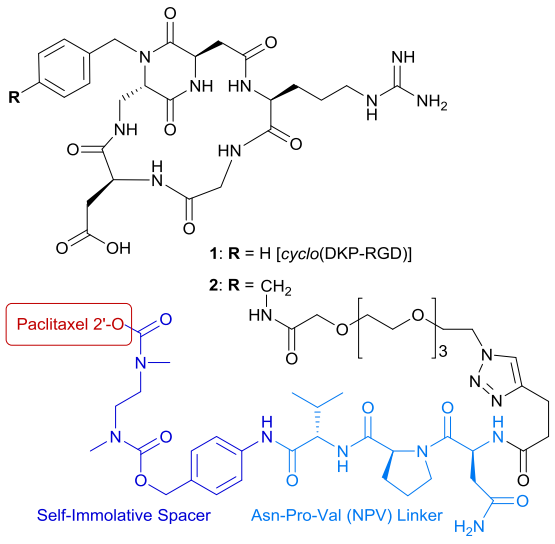

Figure 1. Molecular structure of the $\alpha_v\beta_3$ integrin ligand *cyclo*(DKP-RGD) (**1**) and the conjugate *cyclo*(DKP-RGD)-NPV-PTX (**2**).

In contrast with literature reports on similar ligands,[18] confocal microscopy studies recently showed that ligand **1** accumulates on the surface of $\alpha_v\beta_3$ integrin-expressing cancer cells, without promoting a significant receptor-mediated endocytosis.[19] While this observation may further stimulate the existing debate about the link between the agonist/antagonist behaviour and the internalization of integrin ligands,[20] we focused on the development of non-internalizing conjugates based on ligand **1**. In particular, we recently developed a new

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Electronic Supplementary Information (ESI) available: synthetic procedures, ^{1}H and ^{13}C NMR spectra, HPLC and MS data, procedures for biological and biochemical assays. See DOI: 10.1039/x0xx00000x

SMDC (compound **2** in Figure 1), in which the linker moiety consisted of a substrate of elastase (the Asn-Pro-Val peptide sequence), a serine protease released in the extracellular tumour environment by tumour-infiltrating neutrophils in response to inflammation stimuli.[19] The resulting conjugate exhibited a higher antiproliferative activity against renal cell carcinoma cells 786-O upon addition of elastase in the cell medium, indicating that **2** may be therapeutically efficient against inflamed tumours.

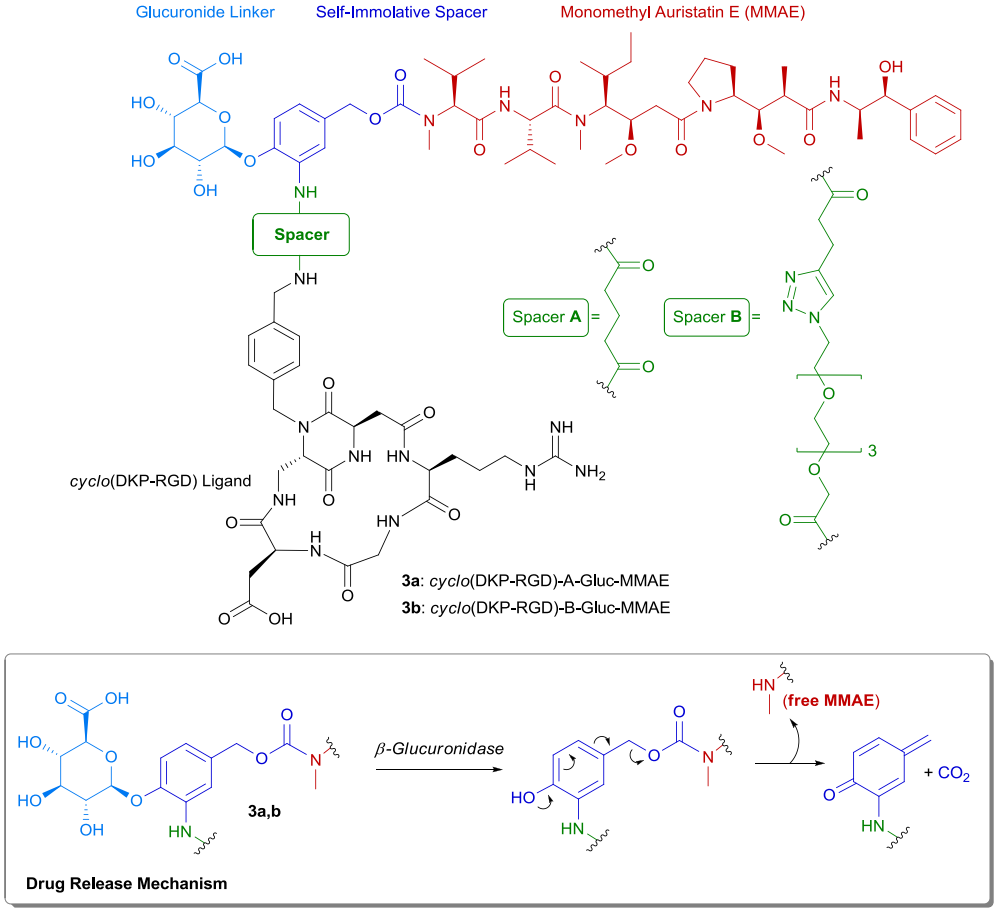
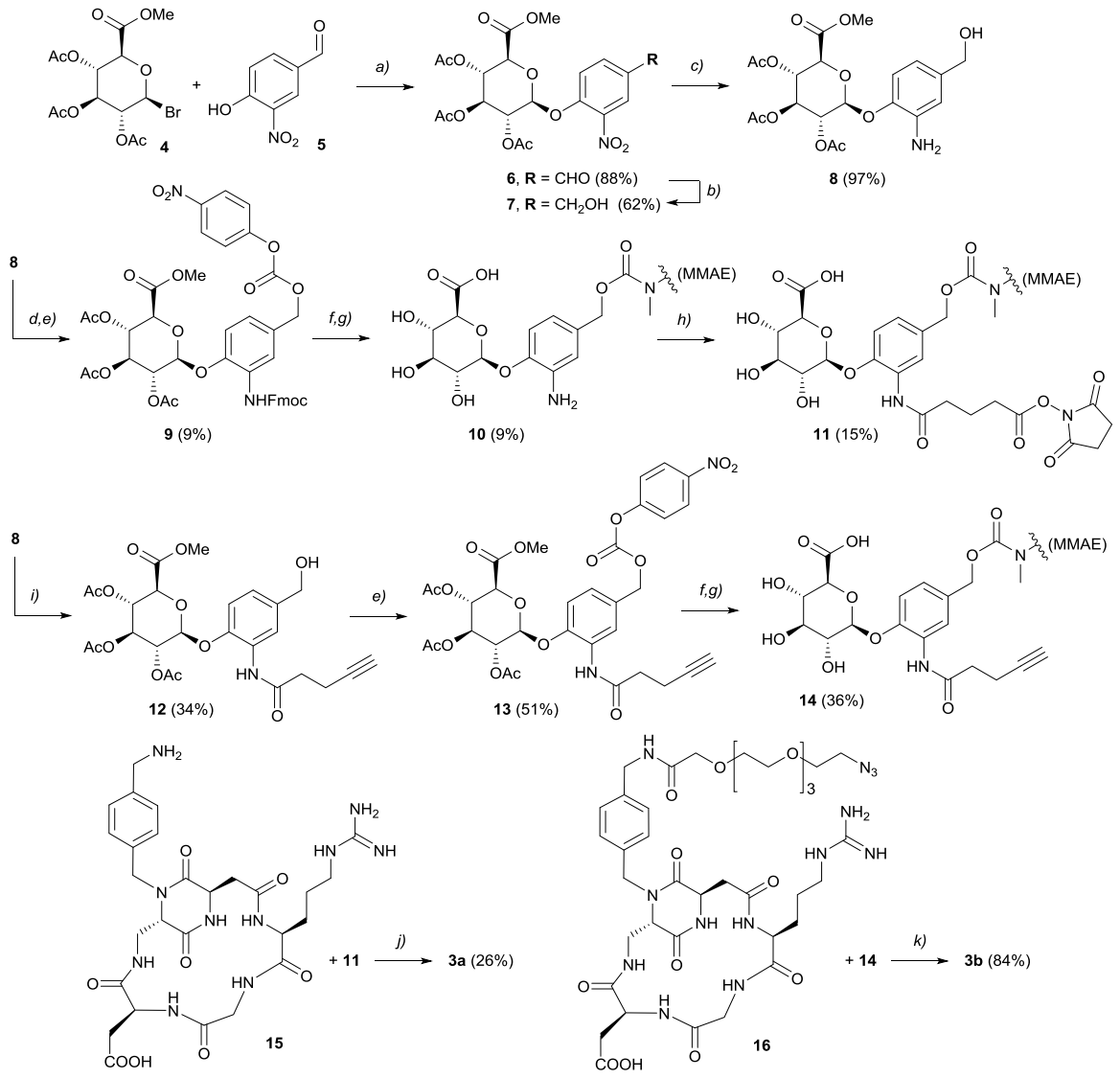


Figure 2. Molecular structures of the $\alpha_v\beta_3$ integrin-targeted conjugates **3a** and **3b**, featuring the *cyclo*(DKP-RGD) peptidomimetic as targeting unit, a glucuronide linker, the anticancer drug monomethyl auristatin E (MMAE) and different spacers (glutaric acid in **3a**, a triazole and PEG4 spacer in **3b**) between the linker and the targeting unit. The mechanism of MMAE release upon linker cleavage mediated by β-glucuronidase is depicted.

Other enzymes may be suitable effectors of non-internalizing SMDCs. For instance, β-glucuronidase is a well-known tumour-associated enzyme, responsible for the hydrolysis of β-D-glucuronic acid residues in glycosaminoglycans. Despite its primary expression in intracellular compartments (lysosomes), β-glucuronidase is often abundant in tumour extracellular areas, shed by apoptotic or necrotic cancer cells as well as by tumour-infiltrating monocytes and neutrophils.[21] For these reasons, β-glucuronidase-responsive linkers have been installed in a large variety of anticancer biotherapeutics, designed to release anticancer drugs both inside[22] and outside[23,24] cancer cells.

Inspired by these studies, we designed two new integrin-targeted conjugates (**3a** and **3b** in Figure 2) equipped with ligand **1**, a glucoronide linker, a self-immolative spacer and the potent microtubule-disruptor monomethyl auristatin E (MMAE), the well-known payload of the marketed ADC Adcetris®. Since the spacer between the ligand and the linker moieties may be crucial for different aspects of the final SMDC assembly (e.g. to modulate the conjugate flexibility, solubility, ligand binding affinity and linker cleavage),[25] conjugates **3a** and **3b** featured two different spacer units (a glutaric acid derivative in conjugate **3a** and a triazole + PEG4 spacer in **3b**). The non-optimized synthetic procedures for the preparation of conjugates **3a,b** are described in Scheme 1. Firstly, a glycosidic bond between bromide **4** and phenol **5** was formed, affording intermediate **6**. Upon aldehyde reduction, the nitro group of **7** was reduced via catalytic hydrogenation. Aniline **8** was used as a common intermediate for the preparation of both final conjugates. For the synthesis of **3a**, **8** was Fmoc-protected and reacted with 4-nitrophenylchloroformate, affording carbonate **9**. The latter was treated with MMAE and HOBt, enabling carbamate bond formation with the secondary amine of the free drug. Basic aqueous conditions allowed protecting group removal, and the resulting free aniline **10** was reacted with di(succinimidyl) glutarate. Following a well-known procedure, the *N*-hydroxysuccinimidyl ester **11** was reacted with the benzylic amine of the *cyclo*(DKP-RGD) ligand **15**[16a] to afford **3a**. Conjugate **3b** was prepared following a similar synthetic plan. However, a significant improvement consisted in the

derivatization of **8** with 4-pentynoic acid, which avoided the installation/removal of protecting groups at the low-reactive aniline moiety and the conjugation of the azide-bearing ligand **16**,[16c,f] via a chemoselective copper-catalyzed Huisgen cycloaddition. The conjugates were purified by semi-preparative HPLC, characterized by high resolution mass spectrometry and lyophilized before biological analysis.



Scheme 1. Synthesis of conjugates **3a** and **3b**. Reagents an conditions: *a)* Ag_2O, molecular sieves, MeCN, overnight; *b)* Silica gel, $NaBH_4$, *i*PrOH/$CHCl_3$, 2.5 h; c) H_2, Pd/C, EtOAc, EtOH, MeOH, overnight; *d)* Fmoc-OSu, *i*Pr_2NEt, DMAP, CH_2Cl_2, 3 h; *e)* 4-nitrophenylchloroformate, pyridine, THF, 3 h; *f)* MMAE, HOBt, *i*Pr_2NEt, pyridine, DMF, 2 h; *g)* LiOH, 1:1 MeOH/H_2O, 2 h; *h)* Di(succinimidyl)glutarate, *i*Pr_2NEt, DMAP, DMF, 3 h; *i)* 4-pentynoic acid, HATU, HOBt, *i*Pr_2NEt, DMF, overnight; *j)* PBS pH 7.5/DMF, 3 h; *k)* $CuSO_4{\cdot}5H_2O$, sodium ascorbate, DMF/H_2O.

The affinity of conjugates **3a,b** for the purified $\alpha_v\beta_3$ integrin receptor was estimated. The conjugates were found to inhibit the binding of the biotinylated vitronectin at nanomolar concentrations, as shown in Table 1. The IC_{50} values of both the MMAE–RGD conjugates **3a** and **3b** were comparable, albeit a bit higher, to that of the free *cyclo*(DKP-RGD) ligand **1**. These data reassured us that the enormous increase of steric hindrance in the MMAE–RGD conjugates did not significantly influence the high affinity for the $\alpha_v\beta_3$ integrin receptor.

Table 1. Inhibition of biotinylated vitronectin binding to the $\alpha_v\beta_3$ receptor.

Compound	$\alpha_v\beta_3$ IC_{50} [nM][a]
cyclo(DKP-RGD)-A-Gluc-MMAE (**3a**)	22.0 ± 9.6
cyclo(DKP-RGD)-B-Gluc-MMAE (**3b**)	76.7 ± 5.8
cyclo(DKP-RGD) (**1**)[15]	4.5 ± 1.1

[a]: IC_{50} values were determined as the concentration of compound required for 50% inhibition of biotinylated vitronectin binding as estimated by GraphPad Prism software. All values are the arithmetic mean ± the standard deviation (SD) of duplicate determinations.

Following the protocol reported by Papot and coworkers,[23] the cytotoxic activity of **3a,b** was examined by incubating the RGD-MMAE conjugates with $\alpha_v\beta_3$-expressing cancer cells, in the presence or absence of β-glucuronidase. This experiment aims at simulating the tumour microenvironment *in vivo*, where a high extracellular expression of β-glucuronidase is expected to promote MMAE release from the non-internalizing conjugates, and the free drug can then enter cancer cells by passive diffusion. Human glioblastoma U87MG[26] and renal cell carcinoma 786-O[19] cells were chosen, and their high $\alpha_v\beta_3$ surface expression was confirmed by flow cytometry analysis (Figure S1 in the Supporting Information).

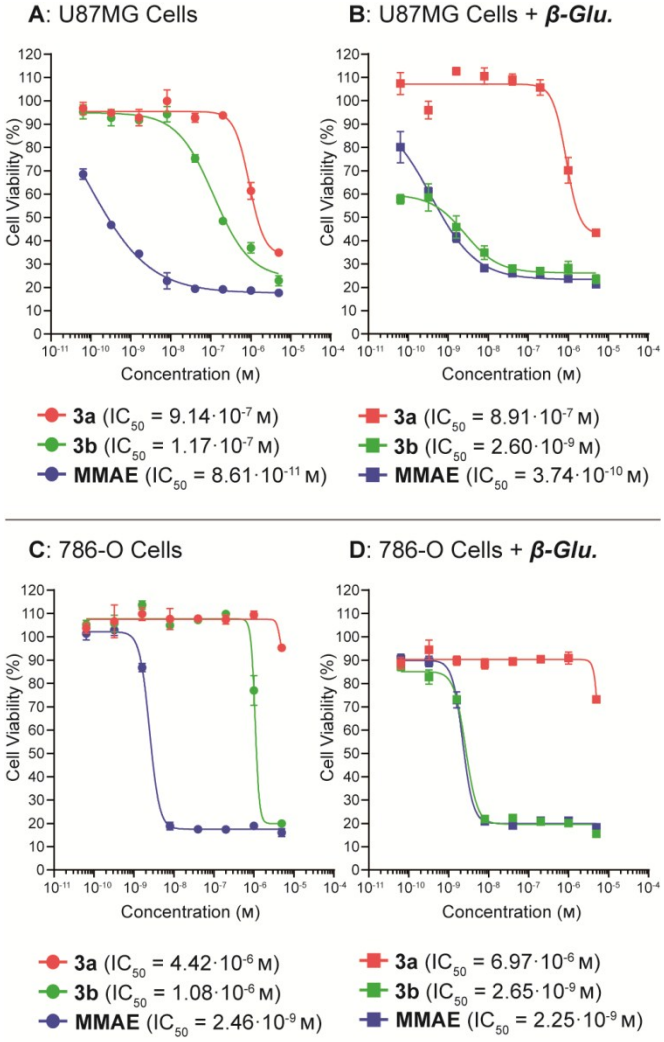


Figure 3. Antiproliferative activity of conjugates **3a**, **3b** and free MMAE in $\alpha_v\beta_3$-expressing human glioblastoma (U87MG) and renal cell carcinoma (786-O) cells, after incubation for 96 hours in the absence (experiments A and C) or presence (experiments B and D) of β-glucuronidase. Data points are averages of 3 experiments. Error bars indicate SDs. Calculated IC_{50} values (M) are listed below each experiment.

As shown in Figure 3A and 3C, in the absence of β-glucuronidase, conjugates **3a,b** proved poorly cytotoxic, whereas free MMAE inhibited cell proliferation at very low concentration (IC_{50} = ca. 86 pM against U87MG and ca. 2 nM against 786-O cells). Interestingly, in both cell lines the activity of conjugate **3b** was found to be a bit higher (IC_{50} 4.1-7.8 times lower) than that of analogue **3a**. On the other hand, the anticancer activity of conjugate **3b** increased dramatically upon enzyme addition, with IC_{50} values in the same low-nanomolar range of free MMAE (Figure 3B and 3D). Noticeably, this enhanced cytotoxicity was not observed for conjugate **3a**, which showed a micromolar activity regardless of the β-glucuronidase addition. Conceivably, the lack of anticancer activity of **3a** can be ascribed to its structural features. In particular, the glutarate spacer (Spacer A in Figure 1) establishes a suboptimal distance between the glucuronide linker and the bulky integrin ligand, affecting the efficiency of the β-glucuronidase-mediated cleavage. On the contrary, the triazole + PEG4 spacer (Spacer B) in conjugate **3b** allows a perfect drug release process. Finally, in the absence of the extracellular enzyme, conjugate **3b** inhibits the proliferation of $\alpha_v\beta_3$-positive cells at submicromolar/low micromolar concentrations (Figure 3A and 3C). This observation confirms the non-internalizing behaviour of the *cyclo*(DKP-RGD) ligand. The modest cytotoxic activity of conjugate **3b** under these conditions may be due to a poor internalization leading to drug release mediated by lysosomal β-glucuronidase.

Conclusions

The design of new conjugates for the selective release of therapeutics at the diseased site represents a highly attractive pharmacological approach for the future treatment of cancer and other indications. However, the modular nature of these products requires a fine tuning of their individual components, from the ligand to the linker, from the drug to the spacers. In this work, we developed a non-internalizing, $\alpha_v\beta_3$-targeted conjugate (compound **3b**), capable of releasing the highly potent MMAE payload upon extracellular cleavage of a glucoronide linker. Conjugate **3b** inhibited the proliferation of integrin-expressing U87MG and 786-O cancer cells at low nanomolar concentrations. These data suggest that **3b** may be therapeutically active *in vivo* against solid tumours expressing both integrin $\alpha_v\beta_3$ (required for the conjugate accumulation at the tumour site) and extracellular β-glucuronidase (responsible for the drug release). From the structural point of view, the use of a long and hydrophilic PEG4 spacer, connecting the integrin ligand to the linker moiety, was found to be fundamental for the overall conjugate efficiency, conceivably due to a diminished steric hindrance around the cleavable glycosidic bond. Once released from **3b** in its active form, MMAE can penetrate the tumour cell membrane by passive diffusion. According to this mechanism, MMAE and other lipophilic drugs (e.g. duocarmycins, maytansinoids) should be considered as ideal payloads, rather than more hydrophilic agents (MMAF, α-amanitin), as it was recently demonstrated for other non-internalizing conjugates.[27] As future perspective, the quantitative biodistribution analysis of the *cyclo*(DKP-RGD) ligand in tumour-bearing mice would unveil the therapeutic potential of this class of conjugates.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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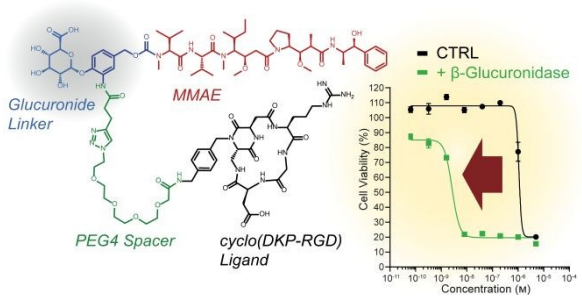

A non-internalizing conjugate targeting $\alpha_v\beta_3$ integrin inhibits the proliferation of integrin-expressing cancer cells in the presence of β-glucuronidase.