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Synthesis and Biological Evaluation of an *iso*DGR-Paclitaxel Conjugate Containing a Cell-Penetrating Peptide to Promote Cellular Uptake

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Dedicated to Prof. Franco Cozzi on the occasion of his 70th Birthday.

Two new Drug Delivery Systems (DDS) *cyclo*[DKP-*iso*DGR]-PEG-4-Val-Ala-PTX (**2**) and *cyclo*[DKP-*iso*DGR]-PEG-4-sC18-Val-Ala-PTX (**3**), containing the *cyclo*[DKP-*iso*DGR] integrin ligand and the cytotoxic agent Paclitaxel (PTX), were synthesized to investigate the influence of a PEG-4 chain and of the sC18 cell-penetrating peptide (CPP) on the cellular uptake and the cytotoxicity of the constructs. A "double click-reaction strategy" was planned, to realize the connection of *cyclo*[DKP-*iso*DGR] and PTX to the CPP moiety. Anti-proliferative bioassays were performed on the $\alpha_v\beta_3$-positive U87 human glioblastoma cell line using a short contact time (15 min) followed by draining, washing of the cells, and re-incubation for 72 h. Compound **3** was significantly more potent ($IC_{50} = 27.6$ μM) than compound **2** ($IC_{50} > 100$ μM), and showed a reduced potency loss with respect to PTX ($IC_{50} =$ 71 nM).

Safety and efficacy are the fundamental features that a drug candidate requires to be approved by regulatory systems. In the quest of new treatments, scientists often encounter very potent and promising new compounds that unfortunately present very high toxicity towards healthy tissues, and low *in vivo* efficacy due to poor pharmacokinetics. Drug-delivery technologies play a pivotal role in overcoming these obstacles,[1] and the selective delivery of drugs to the target pathological cells is one of the most successful strategies used for reducing unwanted side effects.[2] Among the high number of developed systems,[3] antibody-drug conjugates (ADCs) have become a successful approach for the treatment of many neoplastic conditions.[4] In these systems, a potent antitumor drug is linked through a cleavable linker to an antibody that selectively binds a surface receptor overexpressed in cancer cells. Upon the ligand-receptor binding and the cleavage of the linker through external stimulus, the cytotoxic compound is released and accumulated at the tumor site, preventing its distribution into healthy tissues.[5] Subsequently, low-molecular-weight compounds were also exploited as ligands to improve some drawbacks of ADCs, such as costs of antibody production, poor pharmacokinetics, and immunogenicity, giving rise to the so-called small-molecule drug conjugates (SMDCs).[6]

Among the suitable receptors for targeted drug-delivery, integrins, a class of transmembrane proteins, have received special attention because of their overexpression on the cell surface of many types of tumor, such as glioblastoma, renal cell carcinoma, melanoma, and others.[7] These heterodimeric proteins, especially $\alpha_v\beta_3$, and $\alpha_v\beta_5$, are involved in several biological pathways including cell cycle and angiogenesis, and they exploit their signaling pathways after internalization into endosomes upon binding to endogenous ligands. The fact that integrins recognize their natural ligands by the Arg-Gly-Asp (RGD)[8] and *iso*Asp-Gly-Arg (*iso*DGR) sequences[9] opened the way to the design and development of high-affinity integrin ligands containing these motifs for selective targeting of cancer cells.[10] Later on, several SMDCs bearing those ligands bound to cytotoxic agents were reported in the literature.[11] However, the integrin-mediated endocytosis, which would allow the effective delivery of such compounds inside the cells, has not been completely elucidated as different mechanisms of internalization have been proposed. For instance, monomeric integrin ligands would enter the cell through a fluid phase endocytic pathway independent of the $\alpha_v\beta_3$ receptor, while multimeric ligands can cross-link multiple receptors and are internalized via clathrin-mediated endocytosis.[12] Recently, our group has developed cyclic RGD[13] and *iso*DGR[14] peptidomimetics containing a diketopiperazine (DKP) scaffold as low nanomolar and selective $\alpha_v\beta_3$ integrin ligands, which were subsequently conjugated to paclitaxel (PTX),[15,16], α-amanitin[17] and auristatin derivatives[18] through the lysosomally cleavable linker Val-Ala.

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The resulting SMDCs displayed high binding affinity and excellent selectivity towards $\alpha_V\beta_3$; however, a loss of potency compared to the free drugs was observed, probably caused by a poor integrin-mediated internalization process.

For instance, the conjugate *cyclo*[DKP-*iso*DGR]-Val-Ala-PTX (**1**, Figure 1), exhibited a much-reduced potency compared to the free drug (IC_{50} = 927.6 nM for **1** vs 0.64 nM for PTX) but a remarkable selectivity towards U87 human glioblastoma cells ($\alpha_V\beta_3$-positive) compared to the generated clone $\alpha_V\beta_3$-negative U87 β_3-KO (IC_{50} = 4003.0 nM).[16] One of the common strategies used to increase cellular uptake is the introduction of a specific carrier (such as nano-polymers, inorganic vehicles, PEG-, lipid- or peptide moieties) that promotes a different drug internalization pathway (i.e. passive diffusion).[19] In this context, we recently developed a novel drug delivery system (DDS) that combines the selectivity of our *cyclo*[DKP-RGD] ligand, the cytotoxicity of the anticancer drug daunorubicin (Dau), and the cell-penetrating peptide sC18[20] used as drug delivery vehicle.[21] Our results indicated that the cellular uptake of the *cyclo*[DKP-RGD]-sC18-Dau conjugate was mediated by a "kiss-and-run"-like mechanism: the *cyclo*[DKP-RGD] ligand recognizes $\alpha_V\beta_3$-over-expressing cells in a very short contact time (15 minutes), and internalization is mainly mediated by the CPP moiety. Herein, we report our synthetic efforts for the synthesis of two novel SMDC's in which the potent integrin ligand *cyclo*[DKP-*iso*DGR] was connected to the cytotoxic drug Paclitaxel (PTX) introducing specific permeable vehicles in between. In particular, a PEG-4 spacer (compound **2**, Figure 1) and both a PEG-4 spacer and the CPP sC18 (compound **3**, Figure 1) were inserted into **1** to investigate their role in improving the internalization of these compounds through the cell membrane after recognition and binding to the integrin antigen, and eventually increasing the potency of these drug delivery systems compared to the parent *cyclo*[DKP-*iso*DGR]-Val-Ala-PTX (**1**) described above.

PTX has been frequently chosen as a cytotoxic payload, since it can be linked to the SMDC's and easily released as a free drug, without negatively affecting its cytotoxicity. The introduction of a self-immolative spacer, such as *para*-amino-

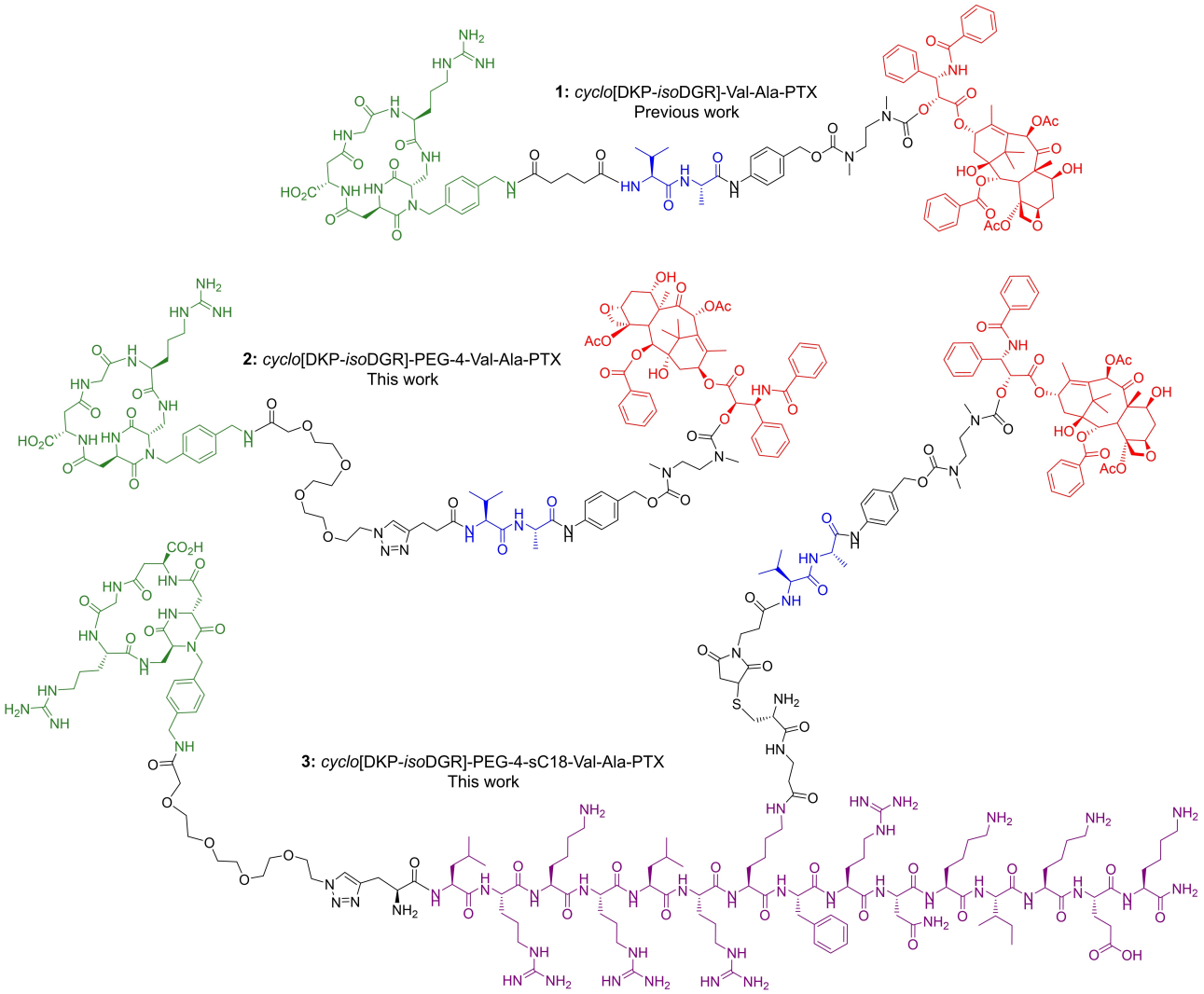
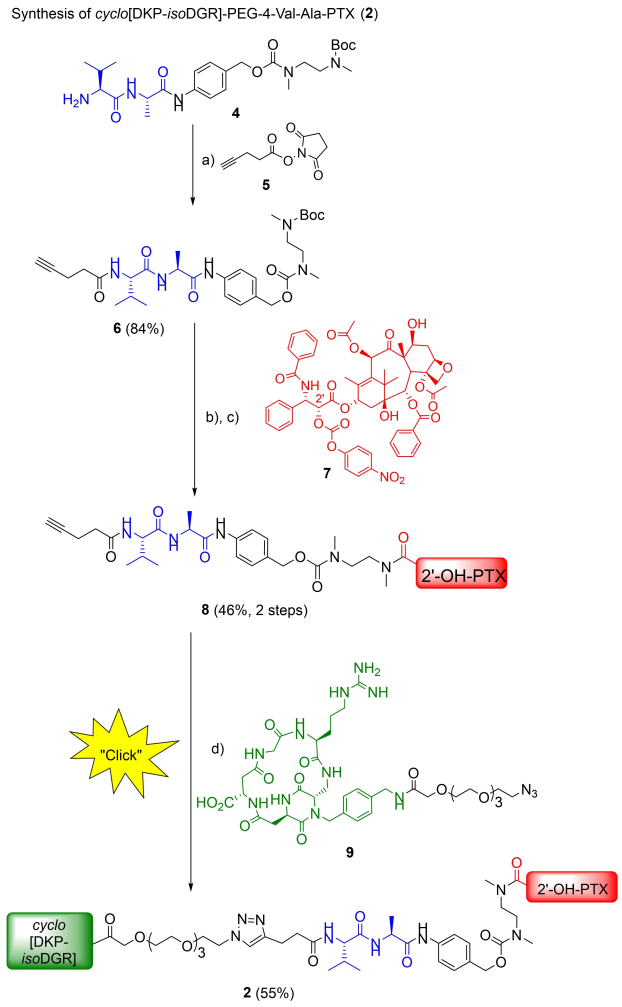


Figure 1. Conjugates *iso*DGR-PTX: *cyclo*[DKP-*iso*DGR]-Val-Ala-PTX **1**, *cyclo*[DKP-*iso*DGR]-PEG-4-Val-Ala-PTX **2** and *cyclo*[DKP-*iso*DGR]-PEG-4-sC18-Val-Ala-PTX **3**. Subunits are depicted with different colors, respectively: cyclo[DKP-*iso*DGR] in green, PTX in red, Val-Ala linker in blue, and the CPP sC18 in purple.

benzyl carbamate (PABC)-*N,N'*-dimethyl-ethylenediamine, between the drug and the enzymatically cleavable peptide sequence helps to realize the detachment without leaving linker vestiges on the PTX molecule.

The synthesis of *cyclo*[DKP-*iso*DGR]-PEG-4-Val-Ala-PTX conjugate **2** was performed as described in Scheme 1. Compound **4** containing the lysosomally cleavable Val-Ala linker and the (PABC)-*N,N'*-dimethyl-ethylenediamine self-immolative spacer was prepared according to a previously reported methodology.[15a] Treatment of compound **4** with 4-pentynoic acid-NHS ester **5** led to alkyne **6** in good yield (84%). Sequential Boc-cleavage in the presence of trifluoroacetic acid and reaction with 2'-(4-nitrophenoxycarbonyl)-paclitaxel **7** gave the carbamate **8** in moderate yield (46% over two steps). Finally, a copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) «click» reaction between **8** and the functionalized ligand *cyclo*[DKP-*iso*DGR]-PEG-4-azide **9** afforded the conjugate **2** in 55% yield (Scheme 1).

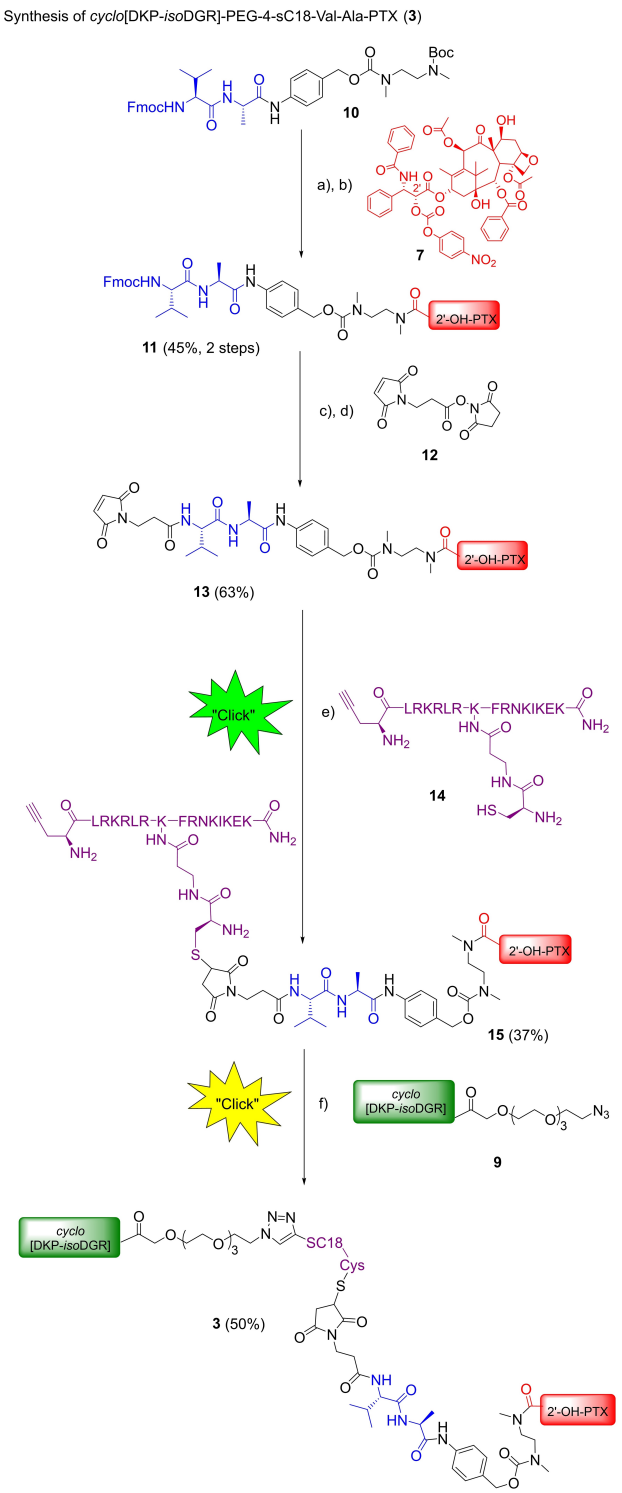


Scheme 1. Synthesis of conjugate **2**. Reagents and conditions: a) 2,5-dioxopyrrolidin-1-yl pent-4-ynoate -NHS ester **5**, iPr$_2$NEt, DMF, RT, overnight; b) DCM/TFA 2:1, RT, 2 h; c) 2'-(4-nitrophenoxycarbonyl)-paclitaxel **7**, iPr$_2$NEt, DMF, 0 °C to RT, overnight; d) *cyclo*[DKP-isoDGR]-PEG$_4$-N$_3$ **9**, CuSO$_4$·5H$_2$O, sodium ascorbate DMF/H$_2$O (1:1), RT, overnight. iPr$_2$NEt = *N,N*-diisopropylethylamine; RT = Room Temperature.

Whereas the synthesis of conjugate **2** was straightforward and similar to other previously reported SMDCs, the introduction of the CPP moiety in **3** required special synthetic efforts. Indeed, in this case, the CPP was acting as a linker itself between the integrin ligand on one side and PTX on the other, and it was necessary to conjugate these two moieties in two different time frames, minimizing the use of protective groups, and in the presence of several reactive functionalities. Looking for an easy strategy to connect the Val-Ala-PABC-*N,N'*-dimethylethylendiamine-PTX to the CPP, the thiol-maleimide "click" reaction caught our attention due to its extended use in bioconjugation transformations.[22] In this reaction, the selective Michael-type addition of a thiol group to the double bond of a maleimide moiety occurs in an aqueous solvent buffered at pH lower than 7.5, to avoid the possible competing conjugate addition of amino groups to the same maleimide. A suitable thiol group was therefore introduced into the sC18 chain through the functionalization of the Lys side chain at position 8 with the dipeptide Cys-β-Ala, whereas the maleimide group was bound to the Val residue of the lysosomal cleavable linker. In addition, the CPP was also functionalized with propargylglycine at the *N*-terminus in order to connect the functionalized integrin ligand *cyclo*[DKP-*iso*DGR]-PEG-4-azide by CuAAC as reported.[21]

This "double-click-reaction strategy" successfully yielded conjugate **3** as detailed in the synthetic pathway shown in Scheme 2. In a first instance, the functionalized peptide sC18 **14** was prepared in multiple steps by automated Solid Phase Peptide Synthesis (SPPS) starting from Rink amide resin (see SI for details). A final manual coupling of the last amino acid, Fmoc-Pra-OH (propargylglycine) afforded the sequence **14**, which is ready for the desired conjugation. In parallel, the Fmoc-protected linker Val-Ala-PABC-*N,N'*-dimethylethylene-diamine **10** was treated with TFA for Boc removal and reacted with 2'-(4-nitrophenoxycarbonyl)-PTX **7** to obtain the carbamate **11** in moderate yield (45%). Further functionalization of **11** with a maleimide derivative **12** led to compound **13** in 63% yield. With all the intermediates in our hands, we could link them together following a "double-click-reaction strategy": the maleimide-containing compound **13** reacted with the cysteine unit of sC18 giving the adduct **15** (37% yield) in a short reaction time (1 h), to prevent retro-Michael deconjugation, while the final linkage was achieved by the CuAAC reaction between the *N*-terminal propargylglycine of **15** and *cyclo*[DKP-*iso*DGR]-PEG-4-azide **9** obtaining conjugate **3** with 50% yield after HPLC purification (Scheme 2).

Compounds **2** and **3** were then evaluated for their activity against tumor cells. Human glioblastoma cells (U87),[23] characterized by a high expression of $\alpha_v\beta_3$ integrin, were used to evaluate the cytotoxic profiles of conjugate **2** and **3** in two different protocols. In the first case, the cells were treated for 72 hours with increasing doses of free PTX and with *cyclo*[DKP-*iso*DGR]-PEG-4-Val-Ala-PTX, and the cell viability was evaluated by MTT assay. An IC$_{50}$ value of 1.2 ± 0.36 μM was measured for compound **2** compared to the 0.3 nM of the free drug, with a substantial loss of cytotoxicity of ca. 4000-fold. The comparison with compound **1**[16] (albeit **1** was tested with a longer



Scheme 2. Synthesis of conjugate **3**. Reagents and conditions: a) DCM/TFA 2:1, RT, 2 h; b) 2′-(4-nitrophenoxycarbonyl)-paclitaxel **7**, iPr_2NEt, DMF, 0 °C to RT, overnight; c) piperidine, DMF, RT, 2 h; d) 2,5-dioxopyrrolidin-1-yl-3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)propanoate- NHS ester **12**, DMF, RT, overnight; e) sC18 **14**, ACN/water (1:1), pH 7, RT, 1 h; f) *cyclo*[DKP-*iso*DGR]-PEG-4-N_3 **9**, $CuSO_4 \cdot 5\ H_2O$, sodium ascorbate, DMF/H_2O (1:1), 40 °C, overnight. iPr_2NEt = *N*,*N*-di*iso*propylethylamine; RT = Room Temperature.

incubation time of 144 hours *vs* the 72 h of these experiments, which showed higher reproducibility) suggests that the PEG-4 moiety does not enhance the permeability of compound **2**. More interestingly, compound **3**, was tested following the short contact times protocol, already employed by us to investigate integrin ligand-CPP conjugates.[21] U87 were incubated in turns with **2**, **3**, and PTX solutions only for 15 minutes, drained, washed, and re-incubated for 72 hours with fresh cell culture media. The results are summarized in Table 1. Paclitaxel, whose uptake is mediated by passive diffusion, showed a marked loss of potency (ca 230 times, *vide supra* and Table 1 entry 1); a similar behavior was also observed for compound **2**, for which an IC_{50} value could not be extrapolated at the tested concentrations. On the contrary, the presence of the CPP moiety in compound **3**, conferred a significantly higher activity with respect to compound **2**. In these conditions **3** is only ca. 350- fold less active than free PTX and favorably compares with the reported values ratios obtained with compounds **1**, **2** (*vide supra*) and other *cyclo*[RGD-DKP] and *cyclo*[DKP-*iso*DGR] conjugates (Table 1).

These results demonstrate that the insertion of the CPP unit is a robust strategy for enhancing the cellular uptake in a "kiss-and-run"-like model, in which the CPP mediated internalization overcomes the inefficient fluid-phase endocytic uptake of the monomeric *iso*DRG integrin ligand,[12] whose role consists mainly in the recognition and binding of the tumor cell-specific antigen.

In conclusion, we reported the synthesis of *cyclo*[DKP-*iso*DGR]-PEG-4-Val-Ala-PTX (**2**) and the synthesis of *cyclo*[DKP-*iso*DGR]-PEG-4-sC18-Val-Ala-PTX (**3**) as suitable compounds for the systematic investigation of the influence of a PEG-4 spacer and a CPP in *iso*DGR-containing conjugates. In particular, the synthesis of **3** involved an innovative "double-click-reaction strategy" for the straightforward incorporation of multiple chemical groups without the employment of several protection/deprotection steps. Furthermore, this strategy allowed the insertion of PTX which can be released without any pendent residues thanks to the self-immolative spacer. The anti-proliferative bioassays showed that the short contact time protocol resulted in a significant enhancement of the anticancer activity of **3** compared to **2**, and a strong improvement of the cell permeability of the conjugate bearing the CPP. This procedure confirmed its suitability to highlight differences in the internalization properties of different compounds and simulate *in vivo* conditions, where rapid clearance of the drugs occurs in the extracellular tumor environment. Further investigations are

Table 1. Evaluation of anti-proliferative activity of conjugate **2** and **3** in U87 cell lines with the "kiss-and-run" protocol.

Structure	IC_{50} [μM][a]
PTX	0.071 ± 0.03
cyclo[DKP-*iso*DGR]-PEG-4-Val-Ala-PTX (**2**)	> 100
cyclo[DKP-*iso*DGR]-PEG-4-sC18-Val-Ala-PTX (**3**)	27.6 ± 4.16

[a] IC_{50} values were calculated as reported in the Supporting Information, from viability curves using GraphPad Prism software. All values are the arithmetic mean: SD of triplicate determinations.

ongoing to evaluate the selectivity profile of conjugate 3 towards cell lines with different integrin expression.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Antitumor agents · Click chemistry · Drug delivery · Integrins · Peptidomimetics

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