αvβ3 Integrin-Targeted Peptide/Peptidomimetic-Drug Conjugates: In-Depth Analysis of the Linker Technology

Alberto Dal Corso, Luca Pignataro, Laura Belvisi* and Cesare Gennari*

Università degli Studi di Milano, Dipartimento di Chimica, via C. Golgi, 19, I-20133, Milan, Italy

L. Belvisi

C. Gennari

Abstract: Covalent conjugation of anticancer drugs to targeting carriers (e.g., antibodies or small molecules) capable of selectively binding to tumor-specific antigens, is emerging as a successful strategy to overcome the drawbacks of traditional chemotherapy. Due to its overexpression on blood vessels of human tumors, $\alpha_v\beta_3$ integrin is one of the most studied receptors of tumor-targeted therapeutics: several peptides and peptidomimetics, bearing the RGD (Arg-Gly-Asp) recognition sequence, have been developed as integrin ligands and linked to different anticancer drugs. The resulting integrin-targeted small molecule-drug conjugates (SMDCs) are able to release the cytotoxic agents upon cleavage of a linker under specific conditions (i.e., hydrolysis, enzymatic action or reduction). Despite the significant efforts made in this field, $\alpha_v\beta_3$ integrin-targeted SMDCs are still far from the clinic. In this review, we survey this approach with a special focus on the different linkers employed and the reported biological activities *in vitro* and *in vivo*.

1. INTRODUCTION

For more than half a century, chemotherapy has been the most exploited strategy for the pharmacological treatment of cancer. This approach consists in the administration of drugs which often interfere with fundamental cellular functions (e.g., DNA replication, cell division). The antitumor efficacy of conventional chemotherapeutics is thus limited by their undesired toxicity to healthy cells and by the development of drug resistance. However, recent progresses made in understanding the molecular principles of cancer growth fostered the development of new-generation anticancer drugs. In particular, the discovery of the altered expression of receptors and enzymes in several tumor cell lines gave rise to the so-called active targeting to cancer: this strategy relies on the use of different cytotoxic or imaging agents covalently linked to a targeting moiety, which binds with high affinity to tumor-specific antigens [1]. Since the 1980s, monoclonal antibodies (mAbs) have been studied as targeting vehicles, owing to their high affinity towards tumor-overexpressed receptors. Intensive research in this field led to the recent FDA approval of the two antibody-drug conjugates (ADCs) brentuximab vedotin (Adcetris®) and ado-trastuzumab emtansine (Kadcyla®), definitely proving the technical feasibility and the therapeutic efficacy of this drug-targeting approach [2]. Nowadays, the number of ADCs in clinical trials has climbed to more than 30, while between 100 and 150 are in the preclinical stage [3]. However, the use of antibodies as drug carriers often suffers from limitations ascribable to their

dimensions (e.g., slow extravasation and difficult penetration into the tumor mass) and to their possible immunogenicity, even in the case of humanized or fully human antibodies [4]. To trespass these limitations, small molecule-drug conjugates (SMDCs) have started emerging as a possible alternative. In this kind of prodrugs, a small receptor binder (i.e., a peptide, a vitamin or substrate analog) is covalently conjugated to a cytotoxic agent. Folate receptor (FR) is the most studied target for SMDCs, due to the capability of folate-conjugates to accumulate at the tumor site and clear from FR-negative tissues. Vintafolide (EC145), a folate conjugate of the microtubule-destabilizing agent desacetylvinblastine hydrazide, is currently being evaluated in the Phase 2b TARGET trial in patients with non-small cell lung cancer, while other promising FR-targeted SMDCs are being developed at preclinical level [5-7]. The structure of SMDCs can be divided in three fundamental portions (Fig. **1**): besides the small-molecule binder (ligand) and the cytotoxic agent (drug), the linker is essential for the conjugate's targeting efficiency.
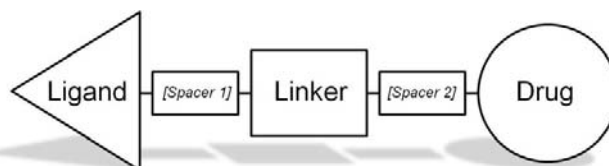

Fig. (1). General structure of a SMDC.

Additional spacers are often present at both sides of the linker (see Spacer 1 and Spacer 2 in Fig. **1**), to modify the conjugate's physicochemical properties (e.g., solubility) or to improve the kinetics of drug release. The cytotoxic drugs

*Address correspondence to this author at the Università degli Studi di Milano, Dipartimento di Chimica, via C. Golgi, 19, I-20133, Milan, Italy; Tel: +39 0250314091; fax: +39 0250314072;
E-mails: laura.belvisi@unimi.it; cesare.gennari@unimi.it

are generally active only after release from the targeting vehicle. For this reason, the linker should be stable in the blood stream, in order to avoid unselective release (with consequent side effects against healthy tissues) and to allow the conjugate's accumulation at the site of disease. On the other hand, the linker should be rapidly cleaved within the tumor environment, so that the drug can fully display its cytotoxic activity against the diseased cells. In general, the mechanism of drug release displayed by the SMDC is highly dependent on the targeted receptor. Most SMDCs (such as folate-drug conjugates) are internalized by the cell through receptor-mediated endocytosis, after ligand binding to the tumor antigen. The internalized conjugate is then transferred to the early endosomes or to the lysosomes [5], even though examples of SMDCs that target non-internalizing receptors are reported [8]. For these reasons, a good understanding of the receptor-mediated internalization mechanism triggered by ligand binding is crucial for the choice of the linker [5, 9]. An intensively studied receptor is integrin α$_v$β$_3$, a heterodimeric transmembrane glycoprotein that is widely expressed on the blood vessels of human tumors, but not on the vasculature of normal tissues. It is commonly accepted that the overexpression of integrin α$_v$β$_3$ is correlated with disease progression in various tumor types, such as breast cancer, glioblastoma, pancreatic tumor and prostate carcinoma [10]. The Arg-Gly-Asp (RGD) peptide sequence has been found to bind with high affinity to the α$_v$β$_3$ integrin. For this reason, several RGD-bearing peptides and peptidomimetics have been investigated as promising carriers for the delivery of

imaging agents [11-13], lyposomes [14-16], and nanoparticles [17, 18]. Among these applications, only radiolabelled RGD peptides have entered the clinical trials so far, for both imaging and therapy [19-21]. Integrin α$_v$β$_3$ has been also studied as target for SMDCs: compounds with different RGD moieties, cytotoxic payloads and linkers have been developed and studied *in vitro* and *in vivo* [10, 22]. The aim of this review is to summarize the efforts made in this field, presenting the RGD-based SMDCs on the basis of the different type of linker technology adopted (see Table **1**) [23].

2. RGD LIGANDS AND α$_v$β$_3$-MEDIATED ENDOCYTOSIS

In 1984 Ruoslahti and coworkers reported that the ability of fibronectin and other ECM proteins to bind cells was ascribable to an Arg-Gly-Asp (RGD) sequence, present on their cell attachment domain [24]. This sequence was found to promote the binding of ECM proteins to integrins, resulting in cell adhesion [25]. Later on, different libraries of oligopeptides bearing the RGD sequence were developed, leading to the identification of low-nanomolar α$_v$β$_3$ binders. In these ligands the RGD sequence was constrained into a cycle, in order to achieve lower conformational flexibility and higher affinity to the integrin receptor [26-28]. The structural rationale for the high observed ligand-receptor affinity was provided by X-ray analysis of the co-crystals obtained from integrin α$_v$β$_3$ and cilengitide, the most well-known integrin ligand [29]. This crystal structure showed an extended conformation of the RGD sequence in the

Table 1.   **α$_v$β$_3$ integrin-targeted peptide/peptidomimetic-drug conjugates.**

Cleavage Type	Linker [23]	Drug	α$_v$β$_3$ Ligand	Ref.
Hydrolysis	*N*-Mannich base	Doxorubicin	RGD4C	[47]
	ester	Paclitaxel	E[*cyclo*(RGDyK)]$_2$	[49, 50]
	ester	Paclitaxel	E[*cyclo*(RGDfK)]$_2$	[51]
	ester	Paclitaxel	*cyclo*(DKP-RGD)	[52]
	ester	Paclitaxel	AbaRGD; AmproRGD	[53]
	amide, hydrazone	CPT analogues	RGD cyclopentapeptides	[57]
	amide, ester	SMAC mimetic	*cyclo*(DKP-RGD), AbaRGD	[58]
	hydrazone	Doxorubicin	E[*cyclo*(RGDfK)]$_2$	[60]
Enzymatic cleavage	D-Ala-Phe-Lys	Doxorubicin	RGD4C	[66]
	Pro-Leu-Gly	Doxorubicin	E[*cyclo*(RGDfK)]$_2$	[70]
	Pro-Leu-Gly and Val-Cit	MMAE	*cyclo*(RGDfC)	[71]
	Ala-Cit	Namitecan	RGD cyclopentapeptides	[74]
	Val-Ala, Phe-Lys	Paclitaxel	*cyclo*(DKP-RGD)	[75]
	Ala-Ala-Asn	MMAE	Non-peptidic α$_v$β$_3$ inhibitor	[77]
Reduction	Disulfide	Camptothecin	*cyclo*(RGDyK)	[82]
	Pt(IV) complex	Cisplatin	Linear RGD and cyclopentapeptides	[86]
	Pt(IV) complex	Cisplatin, DOX	RGD cyclopentapeptide	[87]

binding pocket, with a 9 Å-distance between C-β atoms of the Arg and Asp residues: this folding allows the arginine side chain to interact with two anionic aspartic acid residues in the α-subunit, whereas aspartic acid binds to divalent metal cation in the metal ion-dependent adhesion site (MIDAS) region of the β-subunit [30]. On the basis of these structural requirements, several research groups undertook the development of peptide and peptidomimetic $\alpha_v\beta_3$ integrin ligands [31-34]. These potent integrin binders were originally conceived as antiangiogenic compounds to be directly used in anticancer therapy. However, two recent findings put in question the usefulness of anti-angiogenetics as single drugs [35-37]: the reported paradoxical pro-angiogenic activity of cilengitide under certain experimental conditions [38, 39] and its failure in improving overall survival in patients with newly diagnosed glioblastoma and methylated MGMT promoter status [40]. On the contrary, exploiting RGD ligands as vehicles for different payloads proved to be a useful strategy. In 1998 the first RGD-based SMDC was reported: the DNA-intercalating agent doxorubicin was coupled to an RGD-bearing peptide (RGD4C, isolated by *in vivo* selection of phage display). The RGD-DOX conjugate showed enhanced volume inhibition of human breast cancer xenografts (MDA-MB-435 cell line) in nude mice and lower toxicity compared to the free cytotoxic agent [41]. The Authors inferred that this promising result was due to an enhanced uptake of the RGD-DOX conjugate by the MDA-MB-435 cell line, which features a high level of expression of α_v integrins. However, no details on the conjugate's mechanism of action were discussed.

The variety of endocytic pathways mediated by integrins has been extensively studied [42, 43]. Proteins such as caveolin and clathrin are able to interact with the tails of $\alpha_v\beta_3$ integrin and promote the formation of vesicles that travel to early endosomes. In this subcellular compartments, the receptor can be either driven to late endosomes and lysosomes for degradation, or recycled to the plasma membrane. Integrin endocytosis is known to be involved in different processes, such as cell migration (by detachment of integrins from the extracellular matrix), integrin recycling and activation of different receptors (e.g., VEGFR-2, involved in angiogenesis) [43]. Being the endocytic process fundamental for the SMDC action, the receptor-mediated endocytosis of several RGD-labeled imaging agents has been described in the literature; in most cases, multipresentation of RGD ligands is reported to enhance the internalization of the conjugates into the target cells [44-46]. This observation has been confirmed *in vivo*: biodistribution studies performed in athymic nude mice bearing U87MG glioma xenografts show that a ^{99m}Tc-labeled RGD dimer displayed higher tumor uptake and higher tumor/blood, tumor/liver and tumor/muscle ratios than the corresponding RGD monomer [13]. The late steps of the integrin-mediated endocytosis of RGD-conjugates are fundamental for the SMDC efficiency: unfortunately, only few examples of intracellular localization of RGD-based SMDC have been reported, which will be discussed in the next paragraphs.

3. LINKERS IN RGD-DRUG CONJUGATES

3.1. Hydrolytically Labile Linkers

Several RGD-based SMDCs have been developed in which the linker contains a hydrolytically-cleavable func-

tional group. Ideally, hydrolysis should be slow enough to allow significant drug accumulation at the tumor site and negligible release of the payload into the blood stream. In 2004, Koch and coworkers conjugated a doxorubicin pro-drug (doxsaliform) to the bicyclic peptide RGD4C. The resulting SMDC (Scheme **1**, compound 1) bears a formaldehyde *N*-Mannich base-linker, whose hydrolysis (half-life = 57 min at pH = 7.3; RPMI 1640 cell culture medium) released an active metabolite of the anthracycline drug (compound 2). Unconjugated doxsaliform turned out to inhibit MDA-MB-435 cell proliferation more efficiently than conjugate 1, while doxorubicin showed the lowest inhibition of cell growth in the series (probably due to a lesser membrane permeability compared to both compound 2 and doxsaliform). On the basis of these results and of the evaluation of intracellular uptake by flow cytometry, the Authors concluded that an extracellular linker cleavage takes place in 1, with fast diffusion of the active metabolite (2) into the plasma membrane [47].

A variety of RGD-paclitaxel (PTX) conjugates have been extensively studied *in vitro* and *in vivo*. The microtubule-stabilizing agent PTX has often been linked to RGD ligands through esterification of the hydroxy group in the 2'-position, even though fast hydrolysis of this kind of PTX esters has been described [48]. In 2005, Chen and coworkers evaluated the antitumor activity of a PTX conjugate of the bicyclic peptide E[c(RGDyK)]$_2$ (compound 3, Fig. **2**) against the metastatic breast cancer cell line MDA-MB-435. Antiproliferative assays showed that, although conjugate 3 was about four times less potent than the parent drug, it promoted an enhancement of apoptotic signaling, possibly resulting from a synergy between the drug's cytotoxic action and the RGD-mediated integrin inhibition [49]. In mice, the analysis of a ^{3}H-labeled analog of conjugate 3 showed a higher tumor uptake and longer retention in the integrin-expressing MDA-MB-435 breast cancer model, compared to the free labeled drug. However, no tumor regression could be observed [50]. The stability of the succinyl linker was not assessed and a premature release of paclitaxel can be surmised. A very similar conjugate (i.e., compound 4, Fig. **2**) was extensively evaluated in a recent study by Satchi-Fainaro and coworkers [51]. The stability of conjugate 4 in a glucose phosphate buffer solution at pH 7 was studied over 24 h, revealing a half-life of only ~2 h at 37 °C. As a consequence, a modest integrin-targeting effect was observed in antiproliferation assays against HUVEC only within a very short time exposure (i.e., less than 1 hour). Half-life in the bloodstream is expected to be much shorter, and the inefficacy of this conjugate on an ovarian carcinoma xenograft model (OVCAR-3) was attributed to hydrolysis of the ester bond at the 2′ position of paclitaxel, which causes premature release of the cytotoxic agent and loss of the tumor-homing effect [51]. Our research group developed a RGD peptidomimetic-PTX conjugate (compound 5, Fig. **3**), bearing an ester linker at PTX 2'-position [52]. The stability of this ester was thoroughly studied: contrary to the above mentioned conjugate 4, it was found to be absolutely stable for days in physiological solution, while a half-life of 165 ± 2 min and 143 ± 3 min was assessed at 37 °C in murine and human plasma, respectively. Low nanomolar values were obtained with conjugate
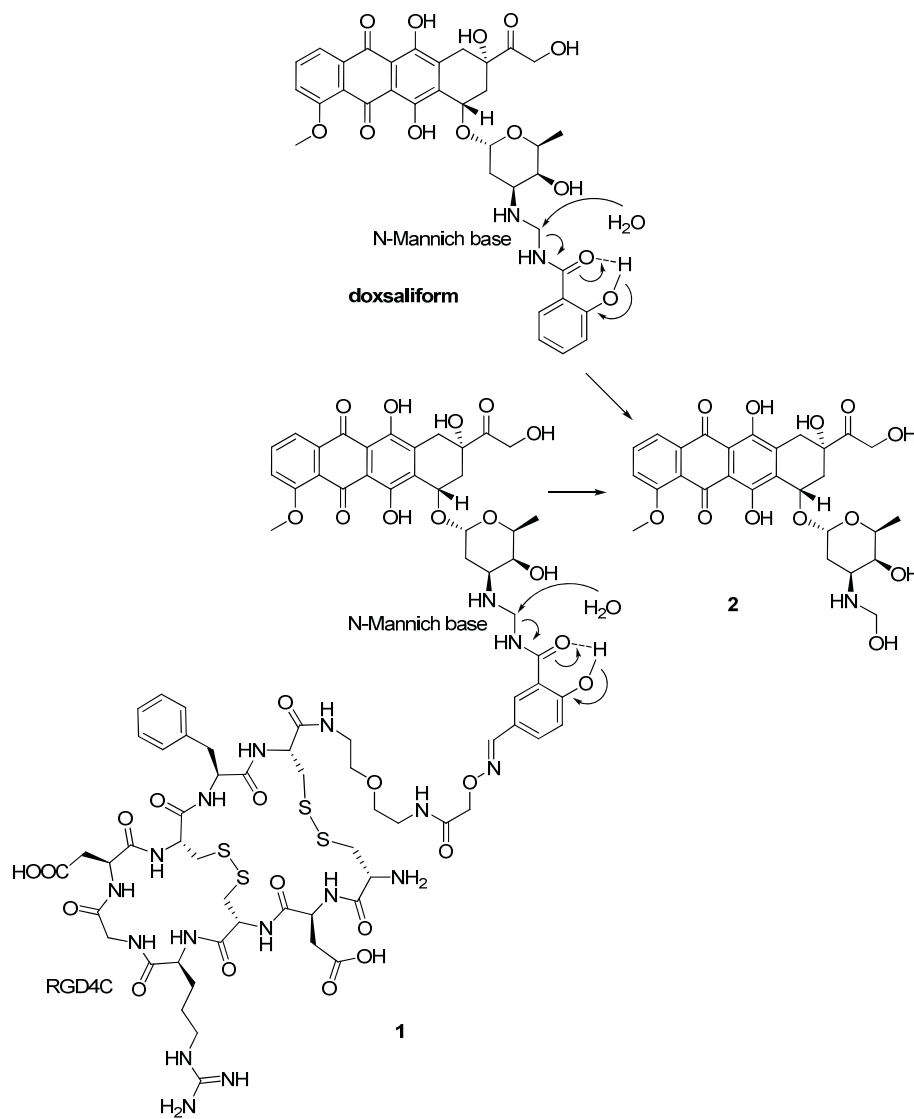
Scheme 1. Hydrolysis of the *N*-Mannich base linker in RGD4C-DOX conjugate 1 and release of doxorubicin active metabolite 2 [47].
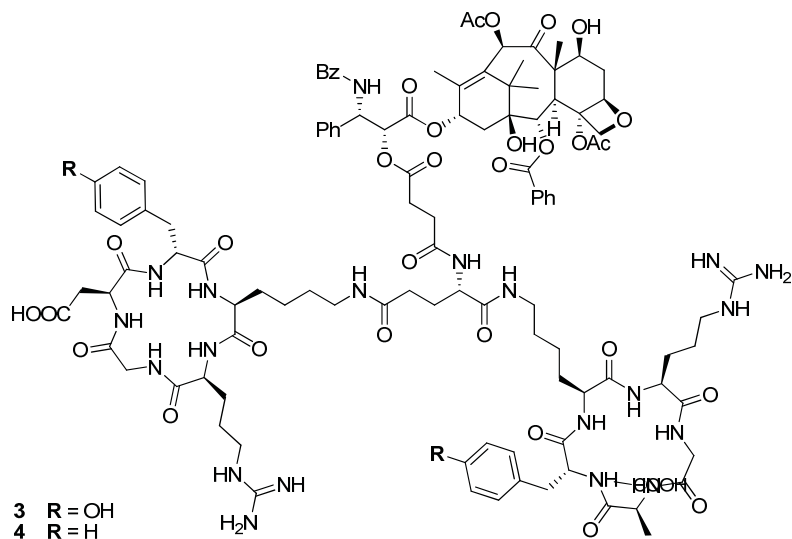
Fig. (2). E[*cyclo*(RGDyK)]₂-PTX (3) [49] and E[*cyclo*(RGDfK)]₂-PTX (4) [51] conjugates.
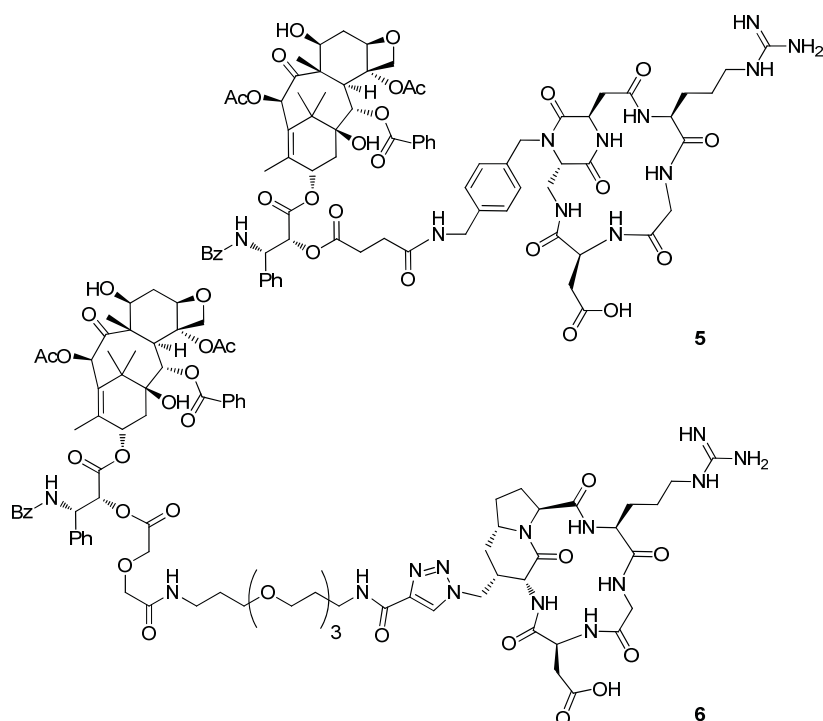
Fig. (3). RGD peptidomimetic-paclitaxel conjugates 5 [52] and 6 [53].

5 in integrin receptor competitive binding assays, comparable to the unfunctionalized ligand. These data proved that the dramatic increase of steric hindrance in conjugate 5, due to presence of the linker bearing paclitaxel through the succinate tether, did not influence the high affinity for integrin $\alpha_v\beta_3$. When administered intravenously to athymic mice xenografted with the ovarian carcinoma IGROV-1/Pt1, compound 5 exerted a stronger effect, in terms of tumor volume inhibition, compared to PTX, despite the lower (ca. half) molar dosage used. Similar results were obtained by the administration to an IGROV-1/Pt1 xenograft in mice of another RGD peptidomimetic-PTX conjugate (compound 6, Fig. 3), developed by Manzoni and coworkers [53].
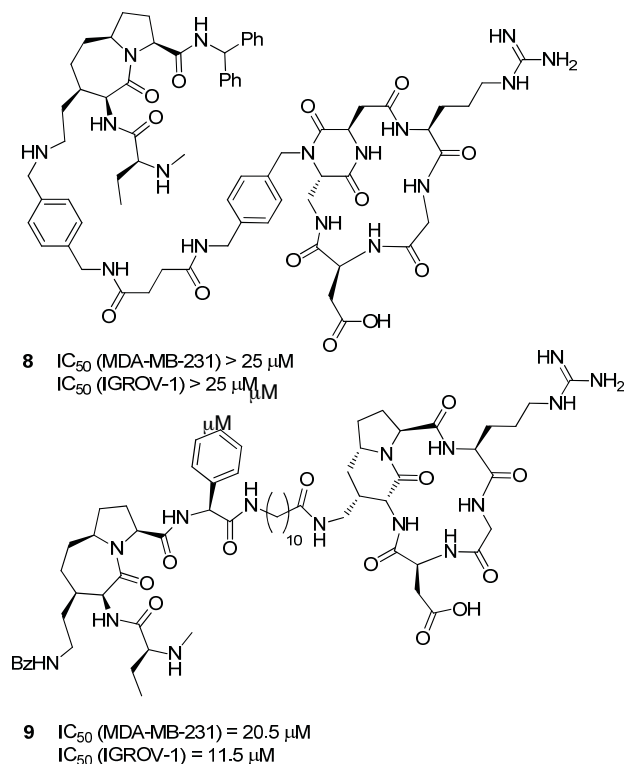
While the readily cleavable ester bonds have been widely employed in integrin-targeted SMDCs, amide bonds have been mostly used as linkers in RGD-conjugates for imaging applications, due to their high physiological stability and synthetic accessibility [54-56].

Indeed, when included as linkers in RGD-based SMDCs, amide bonds often result too stable to allow a substantial release of the cytotoxic payload. For instance, the RGD-camptothecin conjugate 7 bearing an amide linker (Fig. 4) produced a 40% tumor volume inhibition in A2780 human ovarian carcinoma xenograft in nude mice (48 mg/Kg administration). The same 40% tumor volume inhibition was obtained administering a much smaller amount (2.5%) of free drug (0.42 mg/Kg), indicating that the linker between the targeting peptide and the drug in conjugate 7 is too stable [57]. Derivatives of compound 7 with more labile hydrazone linkers will be discussed later in this paragraph. Recently, our research group conjugated two RGD peptidomimetics to a pro-apoptotic SMAC mimetic, installing an amide linker at different sites of the payload (compounds 8 and 9 in Fig. 5). The binding strength of the two separate units was generally maintained by these dual action conjugates. The connection between the two subunits (anchor points on each unit; geometry and stability of the linker) was found to influence the activ-
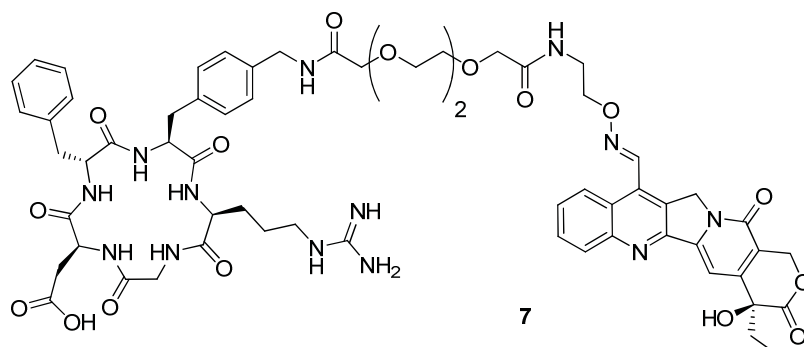
Fig. (4). Camptothecin derivative conjugated to a cyclic RGD-bearing peptide through amide linkers [57].

ity of each portion against its molecular target (integrins $\alpha_v\beta_3/\alpha_v\beta_5$ for *cyclo*-RGD, IAP proteins for SMAC mimetics). As a result, the two conjugates showed significantly different cytotoxic activities *in vitro*. Investigation of their mechanism of action is still in progress [58].

8   IC$_{50}$ (MDA-MB-231) > 25 μM
  IC$_{50}$ (IGROV-1) > 25 μM

9   IC$_{50}$ (MDA-MB-231) = 20.5 μM
 IC$_{50}$ (IGROV-1) = 11.5 μM

Fig. (5). RGD peptidomimetic-SMAC conjugates 8 and 9, with their relative cytotoxicity against MDA-MB-231 and IGROV-1 cells [58].

Among the hydrolytically labile functional groups, hydrazones have been extensively employed as linkers in first-generation ADCs [2, 59], whereas in the SMDC technology their application is limited because the higher solvent exposure often decreases the stability to hydrolysis. For instance, Satchi-Fainaro and coworkers developed a PEGylated doxorubicin (DOX)-E-[c(RGDfK)₂] conjugate (compound 10, Fig. **6**) that can target $\alpha_v\beta_3$ integrin-overexpressing U87-MG cancer cells [60]. Internalization of this RGD-DOX conjugate in the tissues overexpressing integrin $\alpha_v\beta_3$ was detected by fluorescence, while the corresponding RAD-DOX conjugate, unable to bind $\alpha_v\beta_3$ integrin, was not internalized. However, the difference in fluorescence between the RGD-DOX and the RAD-DOX conjugates disappeared in less than 15 minutes, likely due to the fast cleavage of doxorubicin hydrazone linkers. In some cases the hydrazone's hydrolytic stability can be modulated: Dal Pozzo and coworkers suggested that the higher stability under acidic conditions (pH = 5) shown by compound 11 (Fig. **7**, half-life = 25 h) compared to conjugate 12 (Fig. **5**, half-life < 2 min) may be ascribed to π-conjugation of the C=N bond with the aromatic ring of camptothecin. However, no difference in stability could be observed under neutral conditions (pH = 7.4), with both hydrazones displaying half-life of ca. 6 h [57]. The cytotoxicity of conjugates 11 and 12 was tested against PC3 and A2780 cancer cell lines: although these two compounds revealed to be more potent than compound 7 (bearing an amide linker, Fig. **4**), the low stability of the hydrazone linker would presumably result in a premature cleavage and drug release *in vivo*.

3.2. Peptide Linkers

Short peptide sequences that are recognized and cleaved by specific intracellular or cell surface-expressed proteases
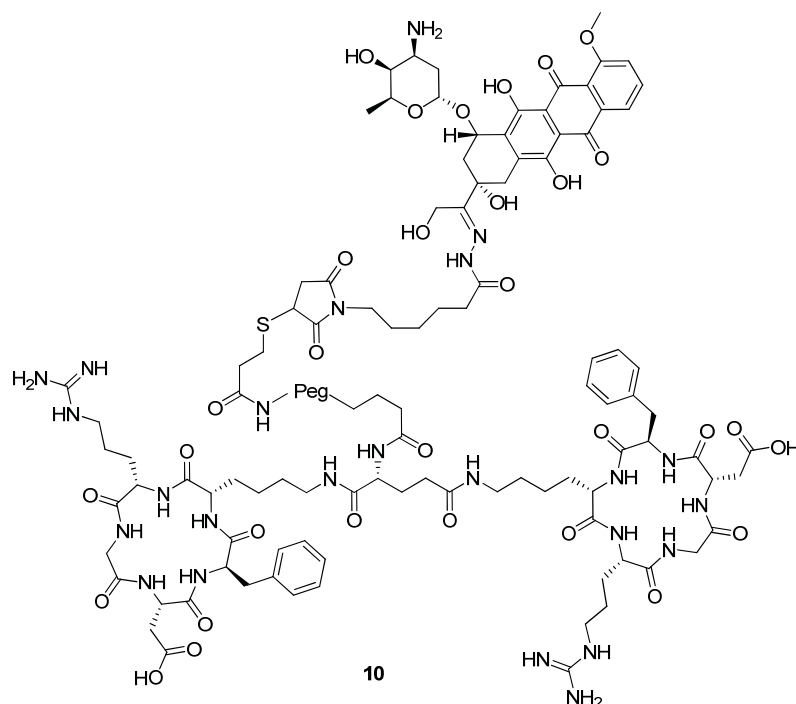
Fig. (6). Structure of PEGylated doxorubicin-E-[*c*(RGDfK)₂] conjugate [60].
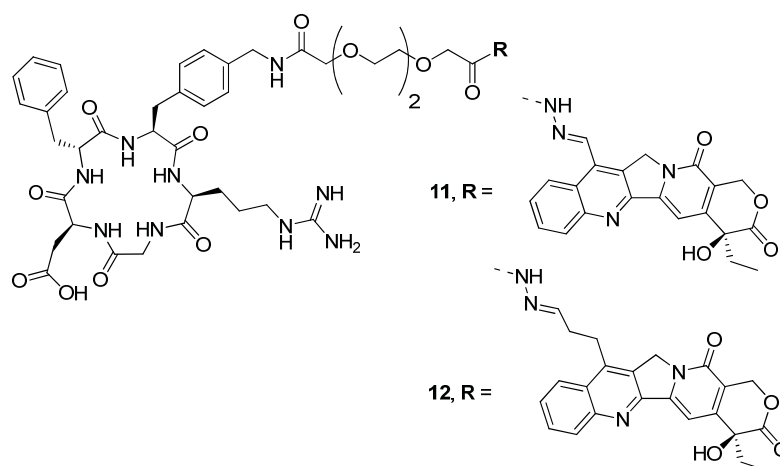
Fig. (7). Camptothecin derivatives conjugated to a cyclic RGD-bearing peptide through hydrazone linkers (11 and 12) [57].

have been often used to trigger the drug release from pro-drugs [61], polymers [62], and ADCs [63].

In these drug-delivery systems, a self-immolative spacer is often present to lower the steric hindrance around the peptide sequence, thus allowing a more efficient enzymatic action. After the cleavage, the spacer undergoes self-elimination through different mechanisms (mainly cyclization and electronic cascade over conjugated π-systems, see Schemes **2** and **4**), releasing the free drug [64, 65]. Peptide linkers generally possess good stability in circulation, thus minimizing unselective release of the drug and related toxici-

ty against healthy tissues. However, their use can be beneficial only if the following requirements are satisfied: (*i*) the SMDC must be able to quickly and selectively reach its target (the tumor cells), where activation is to take place; (*ii*) the activating enzyme, able to cut the peptide linker, must be highly expressed in the targeted cancer cells. In 2002, the first example of RGD-based SMDC with a peptide linker was reported: the integrin ligand RGD4C was conjugated to DOX through the peptide sequence D-Ala-Phe-Lys and the *p*-aminobenzylcarbamate (PABC) residue as self-immolative spacer (compound 13 in Scheme **2**) [66]. The peptide se-
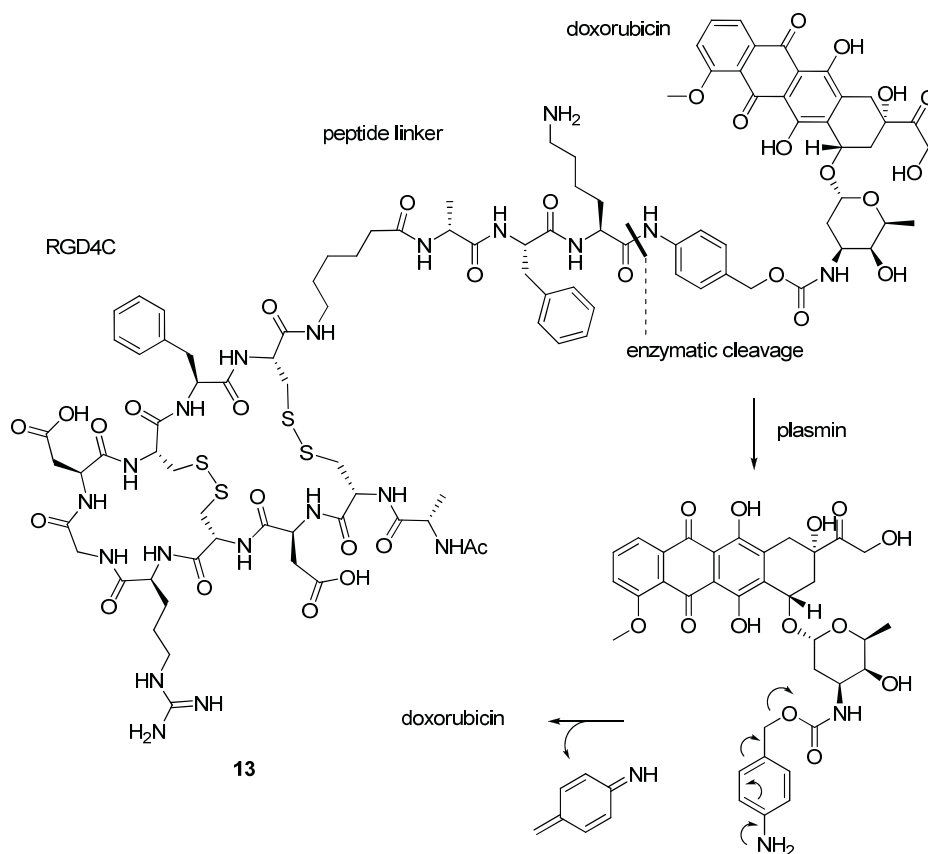

Scheme 2. RGD4C-D-Ala-Phe-Lys-DOX conjugate 13 and release of doxorubicin upon linker cleavage by plasmin [66].

quence is selectively recognized by plasmin, a serine-protease localized on the cell surface and involved in cancer progression [67]. The low cytotoxicity against HUVEC and fibrosarcoma cells HT-1080 exerted by the RGD-DOX conjugate was significantly increased by addition of plasmin, without apparent tumor-targeting effect displayed by the RGD moiety. In addition to plasmin, other cell-surface expressed proteases have been exploited for the activation of RGD-drug conjugates. For instance matrix metalloproteinases (MMPs) are proteolytic enzymes that are secreted by stromal or tumor cells as inactive pro-enzymes and bind to the cell surface to be activated [68]. In particular, the activation of MMP-2 and MMP-9, mediated by $\alpha_v\beta_3$ integrin, is known to play a key role in cell migration, tumor invasion and metastasis [69]. The presence of this "cross-talk" inspired researchers to develop prodrugs able to target both the integrin and the cell surface-expressed proteases. Kratz and coworkers evaluated the *in vitro* and *in vivo* activity of a RGD-DOX conjugate bearing a MMP-2/MMP-9-cleavable linker (the Pro-Leu-Gly peptide sequence). However, in a cytotoxicity assay against HUVEC this conjugate showed similar activity to that of the "control" RAD-DOX conjugate.

This study revealed that the MMP activation is not ascribable to the conjugate's interaction with the integrin receptor, indicating that integrin binding and linker cleavage take place independently [70]. Moreover, since the SMDC binding to the integrin receptor may interfere with the MMP enzymatic activity, unbound RGD-drug conjugates were hypothesized to act as preferential substrates of MMPs [69]. Despite these findings, RGD ligands have been recently employed as tumor homing devices for MMP-cleavable prodrugs. Indeed, a new RGD-monomethyl auristatin E

(MMAE) conjugate (compound 14, Scheme **3**) bearing a MMP-cleavable linker, a D-Arg nonamer (D-Arg9) as cell penetrating peptide (CPP), and a NIR probe (Cy5) was recently developed by Tsien and coworkers [71]. This conjugate showed (*i*) more efficient uptake *in vivo* in MDA-MB-231 breast cancer cells compared to the corresponding RAD-drug conjugate; (*ii*) stronger tumor volume inhibition compared to the free MMAE. In the proposed mechanism of action, the RGD ligand drives the conjugate accumulation at the tumor site. Herein, the MMP-mediated linker cleavage promotes the separation of the D-Arg9 CPP from a polyanionic sequence (D-Glu9), thus allowing the endocytosis of the molecular construct 15 (see Scheme **3**). This results in the intracellular fluorescence of the Cy5 probe and release of MMAE [71]. Substrates of intracellular proteases (e.g., cathepsins and legumain, expressed in the lysosomes) have also been exploited as linkers for RGD-based SMDCs. Interestingly, the lysosomal localization of a cryptophycin-fluorescein-RGD conjugate recently observed by Sewald and coworkers [72] strongly supports the feasibility of lysosomally degradable peptide linkers in RGD-drug conjugates [73]. Dal Pozzo and coworkers tested different dipeptides as linkers for conjugates of RGD cyclopentapeptides with the camptothecin derivative namitecan (NMT, compound 16, Fig. **8**). This study led to the conclusion that, unlike other dipeptides (i.e., Phe-Lys, Val-Cit, Aib-Cit, Tic-Cit), the Ala-Cit sequence possesses remarkable stability in murine blood, together with sufficient cleavability by tumor-associated cathepsins [74]. Moreover, the Authors highlighted the role of the spacer between the linker and the RGD ligand: as a matter of fact, this portion of the conjugate is important to reach an equilibrium between the
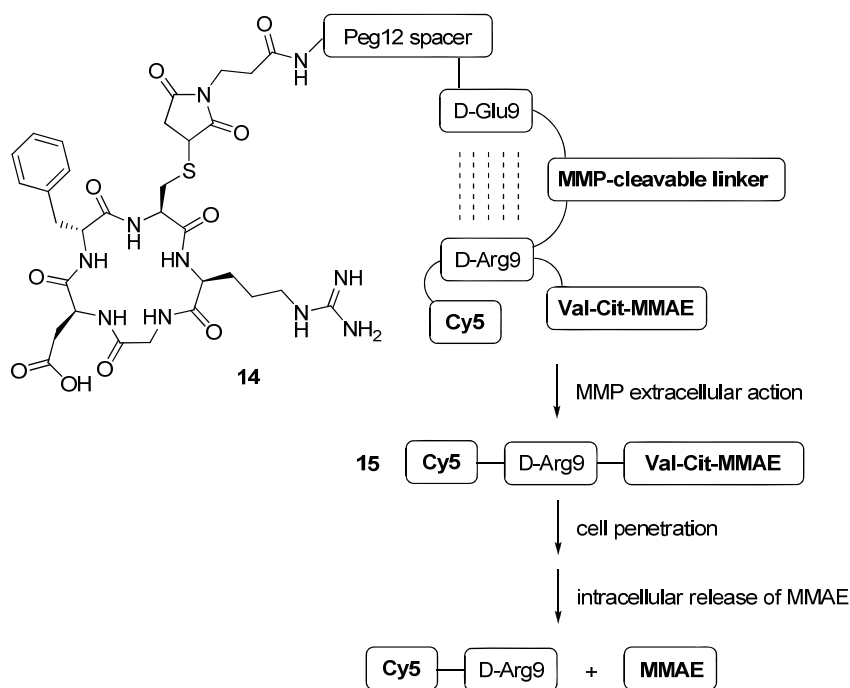
Scheme 3. The theranostic RGD-Cy5-MMAE conjugate 14 and representation of its mechanism of action: the MMP-mediated linker cleavage promotes the activation of the CPP D-Arg9, with subsequent endocytosis of 15. In the intracellular environment, MMAE is released upon lysosomal activation (i.e., cleavage of the Val-Cit linker by cathepsin B) [71].
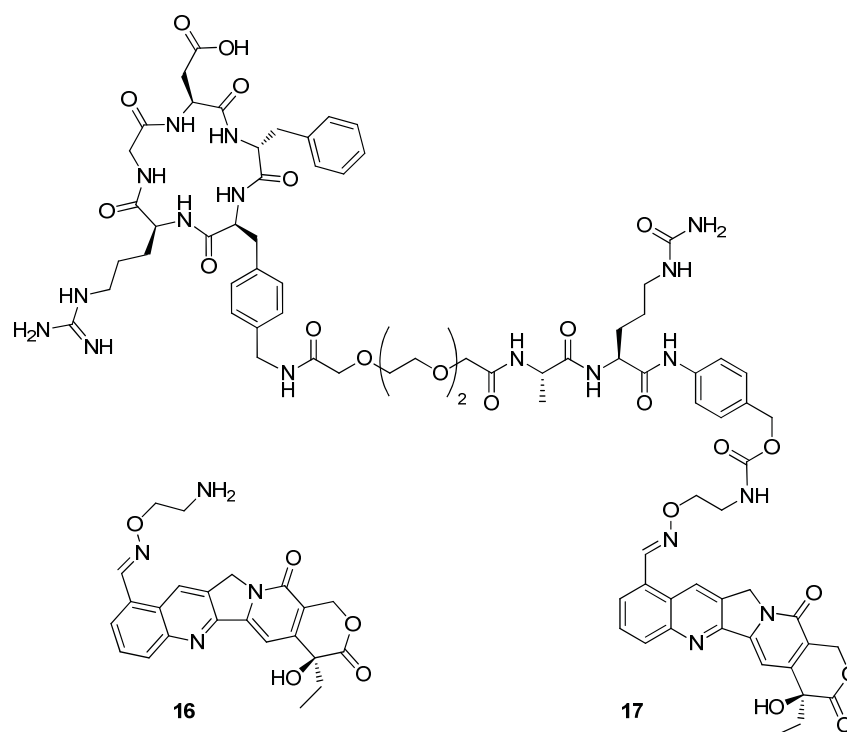
Fig. (8). Molecular structures of the camptothecin derivative namitecan (NMT, compound 16) and that of the SMDCs RGD-Ala-Cit-NMT 17 [74].

compound's hydrophilicity and its plasma stability. The cytotoxicity of the RGD-drug conjugates was tested against A2780 (human ovarian carcinoma) and PC3 (human prostate cancer) cell lines: similarly to the free NMT, the RGD-NMT conjugates (e.g., compound 17, Fig. **8**) inhibited A2780 and PC3 proliferation at nano- and micromolar concentrations, respectively. Unfortunately, the absence of information about the level of integrin expression in these cell lines does not allow to assess the integrin-targeting effect.

A thorough analysis of the integrin-targeting effect displayed by RGD-drug conjugates was recently published by our group. We synthesized two *cyclo*(DKP-RGD)-PTX conjugates (compounds 18 and 19 in Scheme **4**) bearing peptide linkers that were shown to be cleaved by a variety of lysosomal enzymes (mainly cysteine proteases such as cathepsin B). The conjugates' antiproliferative activities were evaluated against two isogenic cell lines, expressing integrin $\alpha_V\beta_3$ at different levels: the acute lymphoblastic leukemia cell line CCRF-CEM ($\alpha_V\beta_3$ −) and its subclone CCRF-CEM $\alpha_V\beta_3$ ($\alpha_V\beta_3$ +) [75]. The integrin-targeting effect was quantified by calculation of the Targeting Index (T.I.), which takes into account (*i*) the SMDC's cytotoxicity against two cell lines expressing the targeted receptor at different levels and (*ii*) the intrinsic selectivity of the "unconjugated" drug payload. The T.I. can be calculated from the observed selectivities S between the tested cell lines (cell line A and cell line B) according to the following equations:

$$\text{T.I.} = \frac{S_{\text{SMDC}}}{S_{\text{free drug}}} \quad (1)$$

$$S = \frac{\text{IC}_{50}\ (\text{cell line A})}{\text{IC}_{50}\ (\text{cell line B})} \quad (2)$$

In our case (see Table **2**), free PTX showed an intrinsic selectivity in favor of the CCRF-CEM $\alpha_V\beta_3$ cells over CCRF-CEM (selectivity factor S_{PTX} = 7.4). While the *cyclo*[DKP-RGD]-Phe-Lys-PTX conjugate 19 showed a modest targeting towards the $\alpha_V\beta_3$-expressing cell line (T.I. = 2.1), its Val-Ala-bearing analogue 18 showed a more pronounced targeting effect (T.I. = 9.0, see Table **2**) [75].

In addition to cysteine proteases, other intracellular effectors have been exploited for the activation of tumor-targeted prodrugs. The asparaginyl endopeptidase legumain is overexpressed in several tumor cells, and this up-regulation is associated with increased tumor invasion and metastasis [76]. Therefore, the legumain's peptide substrate (i.e., the Ala-Ala-Asn sequence) has been exploited by Liu, Sinha and coworkers for the development of anticancer prodrugs [77]. This peptide sequence has been included as linker in a RGD peptidomimetic-MMAE conjugate (compound 20 in Scheme **5**). The Authors analyzed by confocal microscopy the legumain expression in two cell lines expressing $\alpha_V\beta_3$ integrin at different levels: breast carcinoma MDA-MB-435 ($\alpha_V\beta_3$ +) and human embryonic kidney HEK-293L ($\alpha_V\beta_3$ −). Although legumain was detected in lysosome and endosome of both cell lines, in MDA-MB-435 cells the protease was also present on the cell surface, where it associated and co-localized with integrin $\alpha_V\beta_3$. Compound 20 was subjected to antiproliferative assays against the two cell lines, showing higher cytotoxicity against MDA-MB-435, compared to the low $\alpha_V\beta_3$-expressing HEK-293L cell line. The potentialities of this approach were demonstrated also by the conjugate's *in vivo* evaluation: treatment of a MDA-MB-435 tumor model in mice with a dosage of compound 20 three times higher than the maximum tolerated dose of free MMAE resulted in better tumor volume inhibition and much lower toxicity compared to the free MMAE.
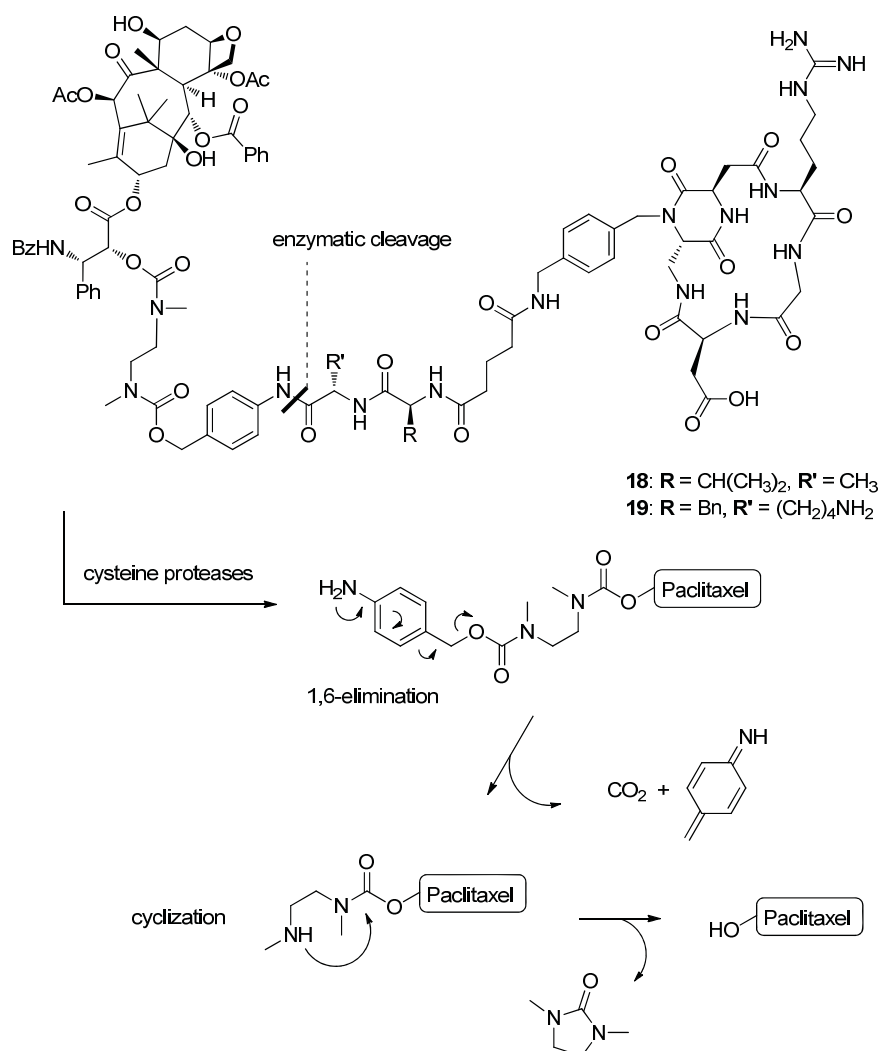
Scheme 4. Molecular structures of the SMDCs *cyclo*(DKP-RGD)-Val-Ala-PTX 18, *cyclo*[DKP-RGD]-Phe-Lys-PTX 19 and their drug-releasing mechanism by a) enzymatic cleavage at the C terminus of the dipeptide linkers; b) 1,6-elimination of the self-immolative spacer; c) release of paclitaxel, occurring by intramolecular nucleophilic attack of the secondary amine onto the 2'-carbamate [75].

Table 2. Antiproliferative activity of *cyclo*[DKP-RGD] conjugates in CCRF-CEM and CCRF-CEM α$_v$β$_3$ after 6 hour-treatment followed by compound washout and 138 hour-long growth in fresh medium. [a] Selectivity (*S*): IC$_{50}$(α$_v$β$_3$ −) / IC$_{50}$(α$_v$β$_3$ +). [b] Targeting Index (T.I.): Selectivity / Selectivity observed with free paclitaxel.

| Compound	IC$_{50}$ (nM)		S[a]	T.I.[b]
	CCRF-CEM (α$_V$β$_3$ −)	CCRF-CEM α$_V$β$_3$ (α$_V$β$_3$ +)		
PTX	155	21	7.4	1
18	5153	77	66.9	9.0
19	535	34	15.7	2.1

3.3. Reducible Linkers

The highly reducing environment of the intracellular compartment and the increased expression of antioxidants in cancer cells (e.g., cysteine, reduced glutathione, thioredoxin, peroxiredoxins, and nicotinamide adenine dinucleotides), inspired the development of delivery systems containing reduction-sensitive linkages [78, 79]. Among these, disulfide linkers have been extensively studied in ADCs [80] and represent the linkers of choice for FR-targeting SMDCs [81]. A theranostic RGD-camptothecin (CPT) conjugate bearing a disulfide linker (compound 21, Scheme **6**) was recently designed by Kang, Kim and coworkers to release CPT in the intracellular compartments and promote a red-shifted fluorescence of a naphthalimide moiety [82].
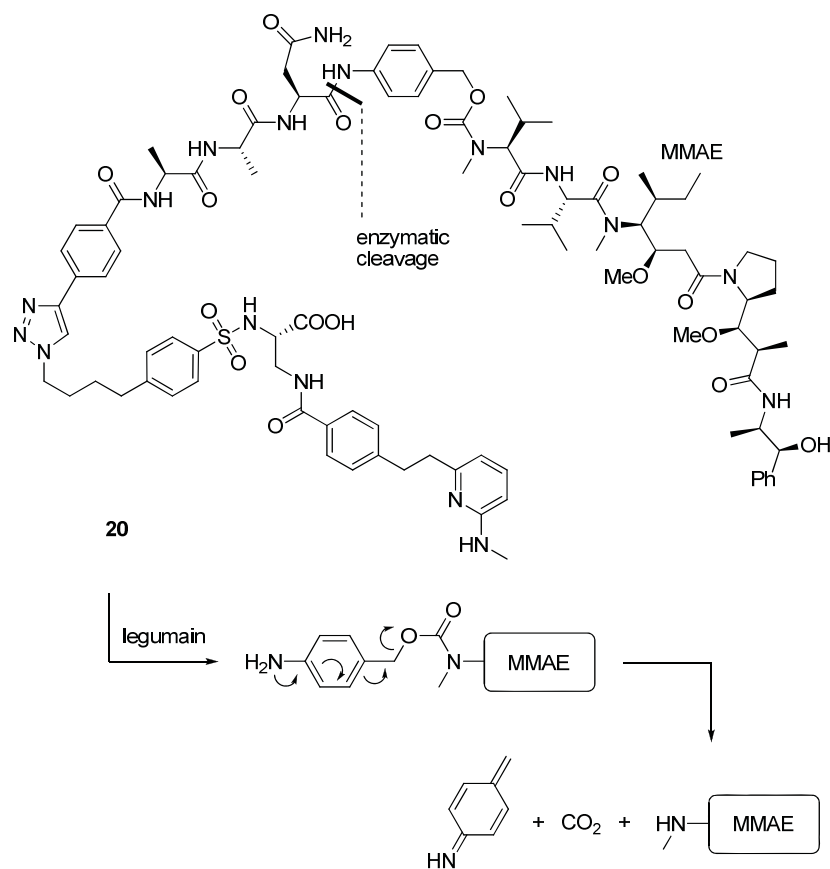
Scheme 5. Legumain-activable RGD peptidomimetic-MMAE conjugate 20 [77], and its drug-releasing mechanism by a) enzymatic cleavage at the C terminus of the Ala-Ala-Asn linker; b) 1,6-elimination of the self-immolative spacer.
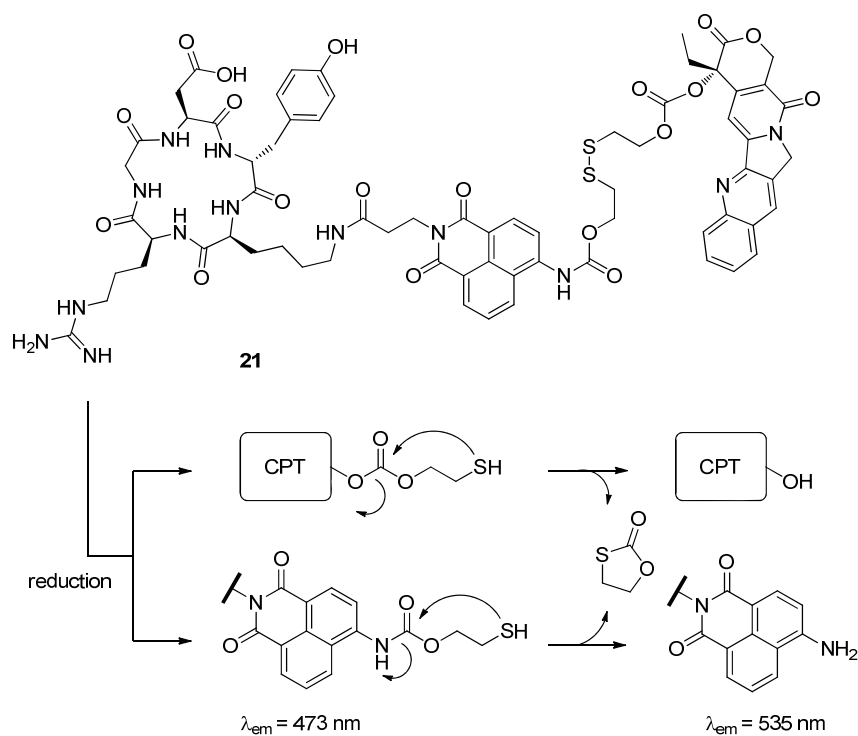
Scheme 6. The theranostic *cyclo*[RGDyK]-camptothecin conjugate 21 [82], bearing a reducible disulfide linker: upon activation by intracellular antioxidants, the free thiol moieties undergo ring closing onto a carbonate and a carbamate functional groups, leading to CPT release and a red-shifted fluorescence emission of a naphtalimide probe.

Confocal microscopy analysis of two cell lines with different levels of $\alpha_v\beta_3$ expression (U87 as $\alpha_v\beta_3$ + and C6 as $\alpha_v\beta_3$ −) showed a stronger conjugate internalization in U87, which was dose-dependently lowered by addition of an endocytosis inhibitor. While the untargeted CPT prodrug was localized in the mitochondria, the SMDC was detected in the endoplasmic reticulum. Moreover, the SMDC displayed a stronger antiproliferative effect than the untargeted prodrug against U87 cells. On the basis of these results, the Authors suggest that the conjugate undergoes an $\alpha_v\beta_3$-mediated endocytosis. However, a negative control test of antiproliferative activity against the low integrin-expressing cell line C6 is not reported. Further evaluation of the conjugates' stability in plasma seems necessary to validate the application of disulfide linkers in the field of integrin-targeting with SMDCs. Indeed, *in vivo* evaluation of disulfide linkers in ADCs revealed that unwanted reduction can occur in circulation [83, 84]. To overcome this limitation, a balance between plasma stability and therapeutic efficiency of the linker system in ADCs has been reached by the addition of aromatic moieties and methyl groups adjacent to the disulfide [83].

In addition to disulfide bonds, reducible metal complexes have been included in tumor-targeting devices to exploit the highly reducing tumor environment. In particular, the reduction of Pt(IV) complexes to Pt(II) has been used for the development of targeted cisplatin prodrugs [85]. In 2008, Lippard and coworkers conjugated a Pt(IV) complex to different peptides bearing RGD and NGR moieties, and tested their cytotoxic activity against a panel of cancer cell lines with different levels of integrin expressions [86]. Although all the conjugates tested revealed to be less potent than cisplatin, all of them showed higher cytotoxicity compared to the untargeted Pt(IV) complex *cis,cis,trans*-[Pt(NH₃)₂Cl₂(succinate)₂] (compound 22, Fig. **8**). Among the targeted prodrugs, the RGD-labeled complexes revealed to be the most active. Interestingly, the conjugation of the platinum complex to two RGD cyclopeptides did not lead to a significant improvement compared to the mono-RGD conjugates. No significant differences were observed in antiproliferative activity against cell lines with high expression of both integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$, compared to cells expressing more $\alpha_v\beta_5$ than $\alpha_v\beta_3$. For this reason, a comparable efficiency of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in the internalization of these RGD-drug conjugates was suggested [86]. A related Pt(IV) complex was recently used by Liu and coworkers to develop an interesting "theranostic"

RGD-drug conjugate: the Pt(IV)-Pt(II) reduction leading to release of cisplatin also promotes the intracellular release of doxorubicin (DOX) from a tetraphenylene (TPE)-labeled RGD cyclopeptide (compound 24, Scheme **7**) [87]. As a result, cisplatin and DOX can exert a synergic effect within the tumor environment where they are released. Moreover, this conjugate was designed to be analyzed by FRET imaging. Due to energy transfer to doxorubicin, TPE's fluorescence is quenched before platinum reduction, whereas the separation between the two fluorophores leads to an enhancement in TPE's fluorescence intensity. The conjugate revealed stronger antiproliferative activity, compared to free cisplatin and doxorubicin, against the high $\alpha_v\beta_3$-expressing cell line MDA-MB-231. Importantly, lower cytotoxicity was observed against the lower integrin-expressing cell lines MCF-7 and 293T. Owing to the presence of fluorophores at both linker's sides, a real-time monitoring of $\alpha_v\beta_3$-mediated endocytosis could be performed: DOX internalization into the cell nuclei was observed over a period of 4 h, while the RGD-TPE moiety was observed in more external intracellular compartments after 1 h of incubation [87].

As can be seen from the examples described above, reducible linkers are emerging as promising tools for the integrin-targeted delivery of cytotoxic agents. However, a deep understanding of the cleavage mechanism and the roles of potential effectors that are involved in the reduction of these linkers is still to be achieved. For instance, the efficient intracellular cleavage of disulfide linkers still needs explanation. Indeed, endocytosis is characterized by a progressive acidification of the intracellular environment and protonated free-thiols are poor nucleophiles for the disulfide exchange reaction. A key role in the cleavage process is thus possibly played by redox enzymes, whose expression may vary significantly from cell to cell [83].

4. PERSPECTIVE

As discussed above, a number of integrin-targeted SMDCs with high structural complexity have been recently developed, in order to achieve an efficient drug cellular uptake and also to better understand the targeted delivery process (e.g., with theranostic devices). Moreover, last generation-RGD-based SMDCs bearing extremely potent cytotoxic agents (e.g., MMAE) have been developed. Although the corresponding free drugs are often too toxic to be administered without a
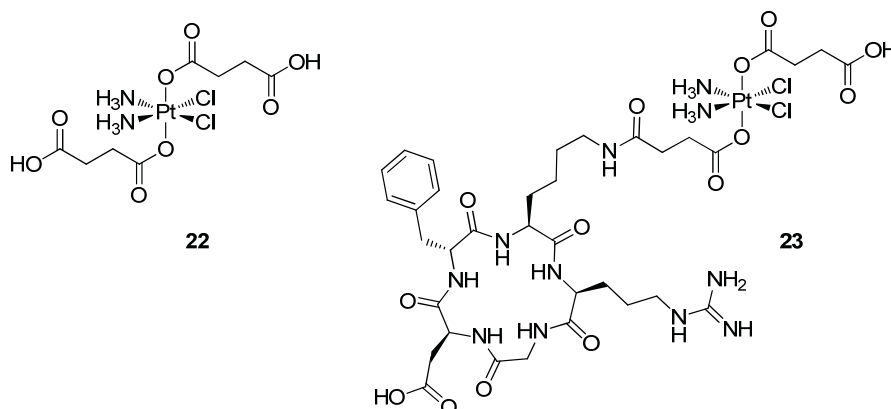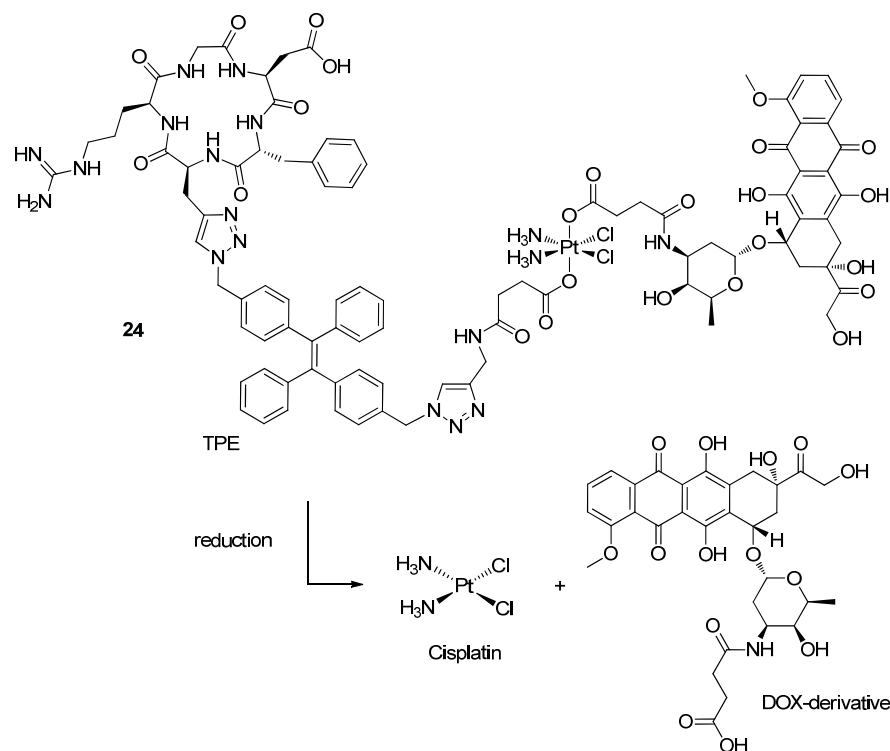

Fig. (9). *cis,cis,trans*-[Pt(NH₃)₂Cl₂(succinate)₂] complex 22 and its derivative (23) with the integrin ligand *cyclo*(RGDfK) [86].

Scheme 7. Tetraphenylene (TPE)-labeled RGD cyclopeptide 24 [87]. The intracellular Pt(IV)-Pt(II) reduction is exploited to deliver cisplatin and a doxorubicin derivative. The separation between TPE and doxorubicin is monitored by FRET imaging, owing to the enhancement of TPE's fluorescence.

Table 3.   Table of the LC_{50} values of peptide compounds KLA and Ir-HRGDH-KLA in A549 and MCF-7 cells [88]. [a] Selectivity (*S*): $IC_{50}(\alpha_v\beta_3-)$ / $IC_{50}(\alpha_v\beta_3+)$. [b] Targeting Index (T.I.): Selectivity / Selectivity observed with free KLA.

Compound	LC_{50} (μM)		$S^{[a]}$	T.I.$^{[b]}$
	MCFF ($\alpha_v\beta_3-$)	A549 ($\alpha_v\beta_3+$)		
KLA	> 250	164.8	> 1.5	1
Ir-HRGDH-KLA	37.7	4.5	8.4	< 5.6

targeting group, conjugation to an integrin-targeting ligand was shown to result in a high drug accumulation at the tumor site and in a better tolerance *in vivo*. Despite these achievements, a general rationalization of the RGD conjugates' mechanism of action at cellular level – and especially of cell internalization – is still missing, and the reported data are sometimes contradictory. For example, while the RGD-CPT conjugate developed by Kim and coworkers was localized in the endoplasmic reticulum of U87 cells [82], the group of Sewald observed the internalization of a fluorescein-labeled RGD-cryptophycin conjugate in the lysosomes of WM-115 cells [72]. To solve these ambiguities, an in-depth analysis of the dependence of the conjugates' anticancer effect on experimental (e.g., the cellular model adopted and the conditions of the biological tests) and structural factors (e.g., the specific $\alpha_v\beta_3$ ligand exploited) is necessary. Another limitation of many recent publications in this field is that no general parameters are provided to allow quantification of the RGD-SMDCs' actual targeting ability and thus to make a comparison between different conjugates possible. We propose the

calculation of the Targeting Index (T.I.) according to Equations (1) and (2) (see Paragraph 3.2) as a universal tool to evaluate the true *in vitro* selectivity of the RGD-drug conjugates. The T.I. values that can be calculated – when possible – for integrin targeting RGD-SMDC are generally quite low, demonstrating that these conjugates are still far from being of clinical utility. For instance, in a recent publication by Fei and coworkers all the parameters required for the calculation of the T.I. were provided: the LC_{50} values displayed against MCFF ($\alpha_v\beta_3-$) and A549 cells ($\alpha_v\beta_3+$) by the membrane-disrupting peptide KLA alone or conjugated to the integrin ligand Ir-HRGDH are reported in Table **3** [88]. The calculation carried out from these data using Equations (1) and (2) results in a modest integrin targeting effect (T.I. < 5.6), lower than the one observed by our group for compound **18** (T.I. = 9.0). Importantly, both values are still far from the targeting skills exhibited by folate receptor (FR)-targeted cytotoxics (e.g., T.I. ~ 200 displayed by the folate-MMAE conjugate reported by Papot and coworkers [89]) and by ADCs (e.g.,

T.I. ~ 2000 displayed by a fully human anti-PSMA mAb conjugated to MMAE [90]).

CONCLUSION

The success of antibody-drug conjugates (ADCs) has proven the efficiency of the "active drug targeting", thus establishing a new paradigm in clinical oncology. At the same time, the intrinsic limitations linked to the use of mAbs (e.g., expensive synthesis and pharmacokinetic issues) have stimulated the interest for small-molecule targeting agents that are – in principle – exempt from these drawbacks. Thus, in the last 20 years the SMDCs have become the object of an increasing interest among researchers. In particular, the integrin targeting SMDCs have been intensely studied for the following reasons: (*i*) some members of the integrin family (in particular integrin $\alpha_V\beta_3$) are overexpressed by a number of tumor cell lines, and thus represent a valuable target for 'smart' drugs; (*ii*) several highly effective peptide and peptidomimetic integrin ligands have been developed throughout the years and can be used for conjugation to drugs. Moreover, the possible synergistic effect between the cytotoxicity displayed by the payload and the RGD-mediated antiangiogenic activity represents an additional, attractive feature of this family of SMDCs. For these reasons, different research groups developed integrin-targeted SMDCs, the most interesting of which have been reviewed above. However, in the papers describing these conjugates, the results of the biological tests are often presented in a form that does not allow a reliable evaluation of the targeting performance. In the cases where the Targeting Index (T.I.) can be calculated from the published data, T.I. values are quite low (more than 1 order of magnitude lower than the T.I. of FR-conjugates and 2 orders of magnitude than ADCs). Therefore, integrin targeting SMDCs can be considered still far from the clinic despite the remarkable progresses that have been realized in the last years. Further investigations of the linker technology, in addition to a more accurate evaluation of the integrin-targeting ability, will possibly lead in the future to the development of new integrin-targeted SMDCs suitable for clinical trials.

LIST OF ABBREVIATIONS

CPT   =   Camptothecin

DOX   =   Doxorubicin

ECM   =   Extracellular matrix

FDA   =   Food and drug administration

FR   =   Folate receptor

HUVEC =   Human umbilical vein endothelial cells

MMAE   =   Monomethyl auristatin E

NIR   =   Near-infrared spectroscopy

PTX   =   Paclitaxel

RPMI   =   Roswell Park Memorial Institute

SMAC   =   Second mitochondrial activator of caspases

TPE   =   Tetraphenylene.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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