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REVIEW ARTICLE

Integrin-Targeted Peptide- and Peptidomimetic-Drug Conjugates for the Treatment of Tumors

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Abstract: ***Background:*** Integrins are heterodimeric cell surface receptors that mediate cell-cell and cell-extracellular matrix adhesion. These molecules play a role in processes such as cell growth and proliferation, differentiation, migration, cell trafficking, besides contributing to angiogenesis and tumor development. Given their biological role, integrins have been proposed as amenable targets in medicinal chemistry. In particular, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ and $\alpha_5\beta_1$, integrins involved in tumor angiogenesis and metastasis, have been the subject of studies aimed at the discovery of novel cancer therapeutics. A large number of peptides and peptidomimetics based on the RGD (Arg-Gly-Asp) recognition sequence were developed in the past two decades as integrin ligands. Though such ligands have not been satisfactory as anti-angiogenic agents, their use as tools to achieve selective tumor targeting of anticancer drugs has been explored.

Objective: In this review, we summarize recent literature and patent applications in which integrin peptidic and peptidomimetic ligands were conjugated to chemotherapeutic agents both with stable or cleavable bonds to achieve tumor targeted drug delivery.

Methods: Relevant recent patents and literature in this field have been considered spanning the search from 2000 to 2016. Literature and patents were examined according to the different classes of cytotoxic drug targeted to integrins.

Conclusion: In spite of the promising features of the conjugates, none of them has entered clinical trials. New efforts are focused on innovative approaches in the field such as the synthesis of new integrin ligands able to target a single integrin type or the employment of nanoparticles based drug delivery systems.

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INTRODUCTION

Integrins are a family of proteins playing a crucial role in linking the cells to the extracellular environment; besides being involved in cell adhesion, they participate in signal transduction as bidirectional signalers because they trigger signals downstream of integrin-mediated adhesion (outside-in signaling) or following binding of intracellular factors resulting in increased ligand affinity (inside-out signaling) (Fig. **1**) [1-3].

Integrins are heterodimeric cell surface receptors that mediate cell-cell and cell-extracellular matrix (ECM) adhesion by connecting cells to the scaffold proteins of the ECM. These integral bidirectional membrane glycoproteins are formed by two non-covalently bound α and β subunits, both class I transmembrane proteins. In vertebrates, 24 different receptors have been recognized, resulting by the combination of 18 α and 8 β different subunits [2], coded by ITGA and ITGB genes, respectively. From a structural point of view, integrins exhibit a large extracellular domain, a transmembrane domain and a small cytoplasmic tail. The extracellular domain of both subunits contributes to form the ligand binding site [4]. Alternatively, integrins can be classified according to their structural features in β_1, β_2, β_3 or α_v subfamilies [5].

Most integrins, including $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$, bind to components of the ECM through the recognition of a common motif consisting of the tripeptide sequence arginine-glycine-aspartate (RGD). Some integrins bind other adhesion receptors on the cell surface, such as intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) or bacterial polysaccharides and viruses components. About a half of the integrin family recognizes this sequence in their adhesion protein ligands. According to their binding capability, integrins can be classified into

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RGD, laminin, collagen and leukocyte specific receptors, although most of them can bind more than one ligand [1].

The integrin extracellular domain exhibits binding sites for divalent cations (Ca^{2+}; Mg^{2+}; Mn^{2+}), which participate in the shift from a close to an open conformation of the integrin molecule (metal-ion-dependent binding) that leads to an increased affinity for the ligand. The intracellular domains of the α subunit are short and not conserved, except for a region next to the transmembrane domain, which is associated with the cytoplasmic tail of the β subunit. Differently, the intracellular domains of the β subunit, are highly conserved and contain two phosphorylation sites which allow the recruitment of adapter proteins, such as talin, that mediate the interaction with the actin cytoskeleton [6] or kindlins, acting as integrin activating proteins through increase of integrin affinity for a ligand [7].

Integrins interact with a variety of ligands present in the ECM, directly or indirectly with multiple substrates on the internal cell side. They can arrange themselves in different conformations that can reflect activation states. When inactive, their state can change due to perturbations occurring inside the cells and affecting the cytoplasmic side of the molecule (e.g., binding of cytoskeletal or signaling factors); these changes can influence the integrin molecule increasing its affinity for ligands. The cytoplasmic tail appears to control the integrin capability to bind a ligand. Among the proteins bound by the cytoplasmic tails, talin plays a crucial role because it allows to separate the α and β subunits, a role underscored by the observation that talin mutations impairing binding to the β subunit prevent integrin activation and adhesion to the ECM. The literature reports different forms of the integrin, i.e., bent, extended and ligand bound forms; such forms reflect respectively low affinity for other molecules, capability to engage other molecules, and ligand binding [8].

Besides their function in mediating cell-cell and cell-ECM interactions as well as their well-defined role in cell motility and invasion, the activation of integrins triggers intracellular signals regulating different cell responses, through the involvement of cytosolic kinases and adaptor proteins. Thus, integrins are important components of pathways which allow the cells to sense and respond to a variety of extracellular signals (e.g., mechanical stress and ligands) derived from the surrounding microenvironment. For this reason, integrins can modulate signaling pathways and gene expression, thereby participating in control of cell survival, proliferation and growth. As a consequence of integrin biological role, integrin defects are linked to a variety of diseases including thrombosis, osteoporosis, inflammation, and cancer [9]. In this regard, multiple studies have shown that cancer cells modulate integrin expression which leads to a more aggressive/invasive behavior of the cancer cell [10]. Moreover, integrin genes can carry mutations, such as gain of function mutations increasing the affinity of the ligand, as described for the beta 1 subunit in squamous cell carcinoma [11]. A variety of sequencing projects and public databases (e.g., Sanger Cancer Genome Project and cBioPortal for Cancer Genomics) have shown the occurrence of mutations whose significance is not completely clear. Since the integrin-binding activity of adhesion proteins can be mimicked by synthetic peptides containing the RGD sequence, binding to integrins by RGD peptides has a therapeutic potential in cancer.

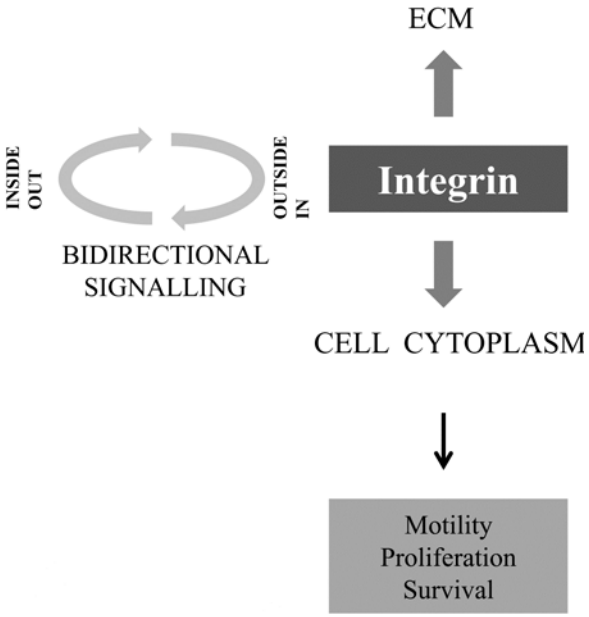

Fig. (1). Schematic representation of integrin signaling. Integrin bidirectional signaling is reported together with the main processes influenced by signaling. Bidirectional signalling refers to those signals that from inside the cell trigger integrin activation promoting a conformation that favours ligand binding (inside-out signalling). Conversely, intracellular signaling can be triggered by ECM (extracellular matrix)-integrin interactions leading to assembly of focal adhesions and reorganization of actin cytoskeleton (outside in signalling).

Here, we review the biological role of integrins with particular reference to cancer and recent literature and patent development with focus on peptide and peptidomimetic-drug conjugates exploitable for the treatment of cancer.

INTEGRINS AND CANCER

Integrin Expression

Integrins are characterized by variable binding properties [5]. Although different integrins share some substrates, the available evidence, in particular the phenotypes of knockout mice suggest very specific functions [12]. Indeed, gene knockouts of integrin in mice show a large range of phenotypes, from normality to early lethality. This observation suggests a very important role for some integrin subunits, and a partially overlapping function during embryonal development.

Integrins are ubiquitously expressed in different cell types, except for some specific molecules related to tissue-specific functions. In particular, β_2 integrin is expressed only in leukocytes and plays a key role in response to infection. Another example of a specific receptor is $\alpha_{IIb}\beta_3$, which is constitutively expressed in platelets, where it binds fibrinogen/fibronectin and regulates the activation and aggregation of platelets [13]. Other integrins, such as $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ are found in neural crest cells, muscles, glial cells, epithelia, osteoclasts and blood vessels during development or angiogenesis, while $\alpha_5\beta_1$ is found in embryo, wound healing tissues and epithelial cells [14]. A wide variety of integrins

contribute to tumor progression through altered expression. In particular, some integrins are up-regulated in different cancers and promote proliferation, invasion and tumor cell survival [2]. Besides being expressed in tumor cells, integrins are expressed in tumor-associated host cells such as endothelial cells and inflammatory cells, in keeping with their established role in angiogenesis.

Integrins in Cancer Cell Motility and Invasion

Integrin functions can modulate various steps of the metastatic process [15]. The early steps require a modification of cancer cell shape to allow migration and interaction with the surrounding tissue, implying epithelial-to-mesenchymal transition (EMT), a process with cell-cell contact loss, cytoskeletal reorganization and acquisition of mesenchymal markers, besides migratory capabilities. Furthermore, integrins control growth and survival under anchorage-independent conditions, during tumor cell escape and intravasation in blood or in lymphatic vessels. For example, in pancreatic cancer and other carcinomas, $\alpha_v\beta_3$ was reported to enhance anchorage-independent tumor growth and to increase lymph node metastases through a mechanisms requiring recruitment of c-Src to the cytoplasmic tail of β_3 integrin, leading to Src activation, Crk-associated substrate phosphorylation independently of activation of FAK (Fokal Adhesion Kinase), a non-receptor tyrosine kinase acting as a major player of β-integrin signaling [16]. Promotion of anchorage-independent growth by β_1 integrin has been related to activation of MAPK (Mitogen-Activated Protein Kinase) signaling [17]. Moreover, $\alpha_6\beta_4$-dependent anchorage-independent growth has been shown to occur through activation of the extracellular signal-regulated kinase 1/2 signaling pathway (ERK1/2) [18]. Finally, the last step of the metastatic process requires cell survival and cancer cell expansion at secondary sites, a condition for which a specific recognition between cancer cells and their surrounding environment is necessary. Integrins, expressed on tumor cells, define the response to new environment signals and initiate metastatic colonization. For example, tenascin C and periostin produced in the metastatic niche act as ligands for different integrins [19, 20]. Moreover, proteolytic fragments of fibronectin have been recently shown to drive the chemotactic affinity of prostate cancer cells to the haematopoietic niche *via* $\alpha_5\beta_1$ integrin, which is highly expressed in disseminated prostate cancer cells [21].

In this context, it has been recently reported that exosomal integrins could prepare pre-metastatic niche by merging with organ-specific resident cells [22].

Importantly, integrins induce the expression of different ECM degrading proteases, such as member of matrix metalloproteinase (MMP) family. In melanoma cells, $\alpha_v\beta_3$ has been shown to bind fibronectin/vitronectin and to induce the expression of MMP-2, whereas the binding of $\alpha_2\beta_1$ with type I collagen has been reported to up-regulate MMP-1, which acts to degrade fibrillar collagens. This degradation is followed by denaturation which uncovers RGD sequences; such RGD motifs act as a ligand for $\alpha_v\beta_3$ integrin, which in turn enhances the expression of MMP-2. Moreover, integrin-ligand binding promotes FAK autophosphorylation at tyrosine 397, which generates a docking site for c-Src Tyrosine kinase (Csk) and Rous sarcoma oncogene cellular homolog (Src) kinase. Csk phosphorylates Src family members at a key regulatory tyrosine in the C-terminal tail and suppresses their activities [23]. These events lead to activation of MAPK/ERK/jun N-terminal kinase (JNK) pathway and consequently to MMP production [24] as a consequence of increased transcription.

The role of integrins in regulation of cell motility and invasion appears to involve proteins belonging to the so-called integrin-adhesome whose main component is FAK [25]. The kinase is enriched in macromolecular complexes termed focal adhesions where adaptor and signaling proteins are recruited to the cytoplasmic tail of integrins following integrin-ECM interaction. By cooperating with the Src kinase, FAK participates in regulating cell adhesion, actin cytoskeleton dynamics cell shape and motility and invasion, in concert with multiple integrin adhesome scaffolding protein such as paxillin [26].

The available evidence supports that Rho family GTPases are required for integrin function acting in concert with E-cadherin [27], underscoring control of the spatial restriction of integrins by modulation of Rho GTPase signaling and altered recycling of other receptors [3].

The recent literature continues to highlight the contributions of integrins that act as RGD receptors to tumor cell aggressiveness, widening the array of mechanisms engaged by integrins. Regarding $\alpha_v\beta_3$, transfer of the protein from tumorigenic to non-tumorigenic cells via exosomes released from metastatic prostate cancer cells has been reported to promote a migratory phenotype [28]. This study appears to be relevant because $\alpha_v\beta_3$ up-regulation has been related to tumor progression and metastatic behaviour in different tumor types [29]. In addition, cells capable to metastasize from the blood stream have been reported to express $\alpha_v\beta_3$ in a constitutively high affinity form, finally indicating that such cells cooperated with platelets to promote extravasation and hematogenous metastasis [30].

A well-established role of integrins is the regulation of tumor angiogenesis [31]. In particular, the contribution of $\alpha_v\beta_3$ integrin to tumor blood vessels formation has been widely documented as it has been provided evidence that the integrin is expressed *de novo* by vascular endothelial cells stimulated by angiogenic cytokines, allowing cell interaction with the remodelling ECM [32]. In addition, the recent literature highlighted a role for $\alpha_5\beta_1$ integrin, corroborated by the evidence that dual $\alpha_5\beta_1/\alpha_v\beta_3$ antagonist display a marked antiangiogenic effect [33].

Integrins, Cell Survival and Drug Resistance

Besides regulating cancer cell motility, invasive capability and participating in the adhesion-dependent control of proliferation, integrins play a role in cell survival [2]. Through their capability to interact with ECM, ligated integrins relay survival signals, whereas unligated integrins promote pro-apoptotic pathways (Fig. **2**). The balance of these signals results in cell survival or apoptosis. Ligated receptors maintain cell survival through different mechanisms involving the phosphatidylinositol 3-kinase (PI3K)-v-Akt murine thymoma viral oncolgene homolog (Akt) path-

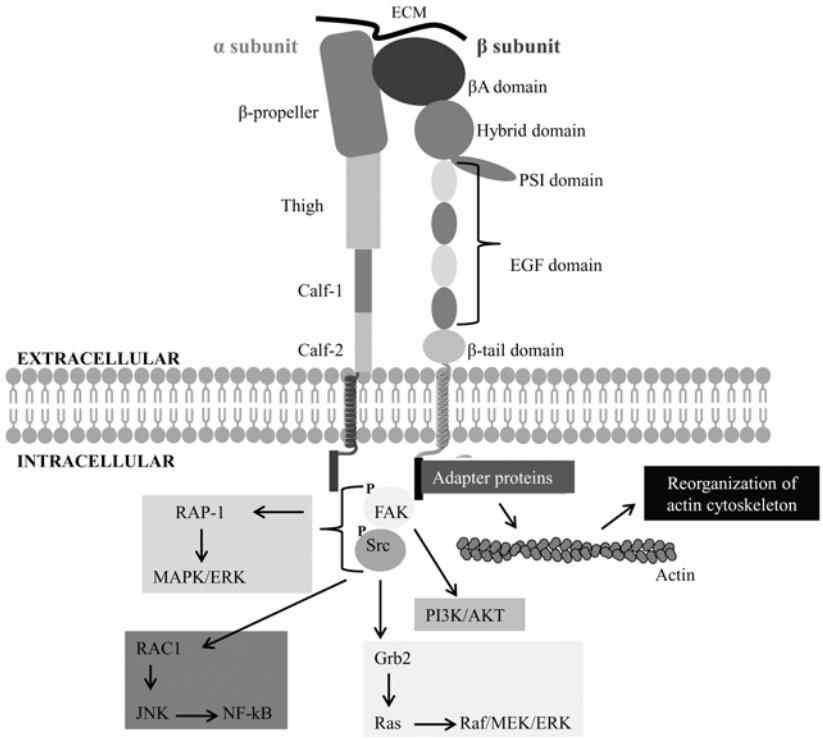

Fig. (2). Schematic representation of the structure of integrins and of integrin-dependent downstream signaling pathways. A variety of pathways are activated downstream of integrin receptors. Abbreviations: AKT: V-akt murine thymoma viral oncogene homolog; ECM: Extracellular Matrix; EGF: Epidermal Growth Factor; P, Phosphorylation; ERK1/2: Extracellular Signal-Regulated Kinase1/2; FAK: Fokal Adhesion kinase; Grb2: Growth Factor Receptor-Bound Protein 2; JNK: C-Jun N-Terminal Kinase; MAPK: Mitogen-Activated Protein kinase; MEK: Mitogen-Activated Protein Kinase Kinase; NF-kB: Nuclear Factor κ B; PI3-K: Phosphatidylinositol 3-kinase; PSI: plexin-semaphorin-integrin; RAC1: Ras-Related C3 Botulinum Toxin Substrate 1/Rho Family, Small GTP Binding Protein Rac1; RAP-1: Ras-Related Protein RAP-1A; Src: Rous Sarcoma Oncogene Cellular Homolog.

way, Integrin-linked kinase, small GTPases of the RAS and Rho families, nuclear factor k-B (NF-kB) signaling, inactivation of p53 and increased expression of pro-survival protein, such as Bcl-2 and filamin A interacting protein (FLIP). A role for unligated integrins in regulating tumor cell survival has been proposed. In particular, unligated receptors on adherent cells recruit and activate caspase 8, activating a process termed integrin-mediated death (IMD) originally described in cells where β_3 expression appeared to be pro-apoptotic to cells in a microenvironment deficient in ligands for β_3 integrin [34]. IMD, a process mediated by the cytosolic domain of β_3 or other integrins [34], is triggered following accumulation and clustering of unligated or antagonized integrins on the cell surface via recruitment and activation of caspase 8, whose loss prevents IMD, whose occurrence thus depends on the local microenvironment and on integrin cell expression [33, 35]. This phenomenon may contribute to explain how in selected environments (e.g., bone marrow) ligands for integrins support survival and metastatic behavior of tumor cells [33].

Integrin signaling acts in concert and in parallel with signaling mediated by growth factor receptors (e.g., vascular epithelial growth factor, VEGF; platelet-derived growth factor, PDGF; and basic fibroblast growth factor, bFGF receptors) to prevent apoptosis. In this context, α_v integrins have been recognized as the main regulators of transforming growth factor β (TGFβ) activation in the tumor stroma [36].

Since integrin signaling occurs through the same downstream pathways activated following activation of growth factor receptors, such as PI3K and MAPK, it is not surprising that integrin expression and activation has been linked to reduced susceptibility of tumor cells to various agents. Indeed, integrins/ECM interactions mediate drug resistance through control of cell death activation [37]. Integrin β_1 has been implicated in resistance to radiotherapy in head-neck cancer [38], to lapatinib and trastuzumab in breast cancer [39] and to erlotinib in lung cancer [40], through the induction of Src and Akt signaling. Moreover, integrins have been shown to promote drug resistance suppressing immune response through the expression of $\alpha_v\beta_3$ integrin on tumor cell surface in response to DNA damage [41].

In a recent translational study carried out in acute myeloid leukemia, the expression of ITGB3 which codes for the β3 integrin subunit was correlated with prognosis of acute myeloid leukemia patients [42]. Moreover, in cellular models, $\alpha_v\beta_3$ activation was shown to enhance β-catenin activation through PI3K/Akt/Glycogen synthase kinase-3 beta (GSK3β) pathway in association with decreased sensitivity to sorafenib [42].

Some recent research articles provide new insights into the role of integrin in mechanisms that may be involved in sustaining tumor aggressiveness. For example, a novel function for αintegrins has been recently reported in a study showing negative regulation of immunogenic cell death by

Table 1. Pattern of Expression of Cancer-Related Integrins [a].

	Distribution	
Integrin	**Normal Cell/Tissue**	**Tumor**
$\alpha_1\beta_1$	Smooth muscle, T cell, endothelium, hepatocyte	Breast, colon, lung, melanoma
$\alpha_2\beta_1$	Epithelium, endothelium, leukocytes, platelets	Breast, colon, kidney, lung, melanoma, skin
$\alpha_3\beta_1$	Epithelium, endothelium	Breast, colon, kidney, lung, melanoma, ovary, skin
$\alpha_4\beta_1$	Leukocytes	Breast, colon, melanoma, skin
$\alpha_5\beta_1$	Endothelium, platelets, hepatocytes, lymphocytes	Breast, colon, kidney, lung, melanoma, ovary, skin
$\alpha_6\beta_1$	Most cells, platelets, epithelium, endothelium	Breast, colon, kidney, lung, melanoma, skin
$\alpha_v\beta_1$	Fibroblasts, osteoblasts	Breast, kidney, lung, ovary, skin
$\alpha_v\beta_3$	Osteoclasts, endothelium, fibroblasts	Breast, colon, kidney, lung, melanoma, skin
$\alpha_v\beta_4$	Neurons, fibroblasts, epithelium	Breast, colon, melanoma, skin
$\alpha_v\beta_5$	Pancreas, fibroblasts	Kidney, lung, skin
$\alpha_v\beta_6$	Epithelium	Colon, ovary, skin

[a] Modified from Mizejewski GJ. Role of integrins in cancer: survey of expression patterns. Proc Soc Exp Biol Med. 1999;222:124-38.

suppressing presentation of cell surface calreticulin, which represents the prophagocytic signal for macrophages. In particular, integrins were shown to exhibit an inhibitory effect on surface calreticulin, likely due to reduced cytosolic to surface translocation [43]. Such events may impact on host capability to counteract tumor development. Concordantly, recent studies show that integrin β3 activation also regulates the balance between antitumor and protumor immune cells through effects on Signal Transducer and activator of Transcription 6 (STAT6)/ Signal Transducer and activator of Transcription 1 (STAT1) signaling [44]. Together with evidence that antagonizing β3 integrin [44] increases immunosuppression in cancer, these results raise some concerns on the therapeutic efficacy of integrin antagonists. However, these observations may also prove helpful to interpret the clinical trial results.

Integrin Endocytosis and Recycling

An additional layer of complexity of integrin biology is represented by their endocytic trafficking which has been implicated in signaling and in regulation of cell migration, likely by Rho GTPase signaling and by alteration of trafficking of other receptors [3]. Integrin trafficking occurs through clathrin-dependent mechanisms implicating conserved motifs recruited to clathrin-coated structures through various adaptor proteins (e.g., Numb; adaptor protein complex 2, AP2; disabled homolog 2, DAB2). Among them, DAB2 has been reported to control endocytosis of β_1 integrins [45]. However, integrins have also been shown to be internalized by other mechanism not involving clathrin, for example via caveolar endocytosis. After endocytosis, internalized integrins move to early endosomes and recycle back to the plasma membrane or to late endosomes and lysosomes for degradation or recycling [3]. The biochemical details of these processes have been deeply addressed elsewhere [46].

From Integrin Biology to Peptide- and Peptidomimetic-Drug Conjugates

The wide knowledge of the biology of integrins has provided the rationale to approach the design of peptide and peptidomimetic of the RGD sequence bound by cancer-relevant integrins, in an attempt to exploit selective and specific binding to deliver potent cytotoxic agents to tumor cells, thus sparing normal cells (Table **1**). In this view, integrin peptide and peptidomimetic are instrumental to accumulate the payload in tumors, mimicking the physiological internalization of integrins via clathrin-dependent mechanisms as well as via macropinocytosis and caveolae. The former mechanisms contribute to trafficking of active ligand bound and inactive unbound integrins to early endosomes, and recycling of inactive integrins to the plasma membrane. Active bound integrins are directed to late endosomes and lysosomes followed by recycling to the plasma membrane or degradation [3, 26]. In the case of peptide and peptidomimetic-drug conjugates, these processes provide a strategy to internalize the payload with subsequent release of the cytotoxic drug in the tumor cells. In fact, the drug diffuses from the lysosomes to reach its target, and also cells in close proximity to those internalizing the conjugates can be killed by bystander effects, mainly due to the diffusion of the free drug into surrounding cells [47].

Integrin-Targeted Drug Delivery

Due to the paramount importance of integrins in the genesis and development of many pathologies and primarily in cancer, great efforts have been devoted to the design and synthesis of integrin ligands [9, 48, 49]. Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$ and more recently, $\alpha_v\beta_6$, involved in cancer progression and metastasis, have been the subject of research studies aimed at the development of peptidic, peptidomimetic and

non-peptidic ligands [50-53]. A large part of these ligands are based on the RGD motif, recognized by Ruoslahti *et al*. as responsible for the interaction between integrins and ECM proteins [54, 55], as subsequently confirmed by the X-ray analysis of co-crystals with different integrins [56-59]. In particular, the structural analysis of the co-crystal between integrin $\alpha_v\beta_3$ and the well-known integrin ligand Cilengitide allowed to identify the binding motifs involved in the interaction. According to the published structure, the binding with the ligand takes place at the junction between the alpha and beta subunits. The arginine residue binds to a negative charged region formed by two Asp residues in the alpha subunit through its guanidinium group meanwhile the aspartic acid is involved in an electrostatic interaction with the divalent metal cation of the metal ion dependent adhesion site (MIDAS) of the beta subunit [60]. These interactions are generally conserved in all the principal RGD-binding integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, $\alpha_v\beta_6$), but differences in the binding pocket size and in the residues involved in the binding have allowed the design of specific ligands for each protein [61].

However, despite encouraging pre-clinical results, none of these high affinity integrin ligands, originally conceived as antiangiogenic drugs, has been able to reach the market as anticancer therapeutic, including the cyclic pentapeptide Cilengitide (*cyclo*[RGDf(N-Me)V]) that has recently failed a Phase III clinical trial for the treatment of glioblastoma, though it is currently undergoing other Phase II clinical trials for the treatment of different cancers [62, 63].

More encouraging is the picture depicted by the use of integrin ligands as targeting moieties able to drive imaging agents or cytotoxic drugs to integrin rich tumor sites [62, 64-71]. In the case of conjugates with cytotoxic drugs, of particular relevance is the ability of integrin ligands not only to drive the drug to the therapeutic site, but also to exploit the documented ability of integrins to mediate endocytosis through different pathways in order to accumulate the drug inside the cells [3]. Once internalized, most cytotoxic drugs need to be released by the conjugated construct to exert their action. In most cases, conjugates are thought to be cleaved by esterases. However in some studies, specific bonds were designed to be broken by the acidic pH of subcellular compartments or hydrolyzed by specific enzymes that are overexpressed in the tumor microenvironment.

Despite the large amount of the synthesized integrin ligands, only a limited number of them present in their structure functional groups suitable for chemical conjugation while preserving high binding affinity. Among the most studied are the cyclopentapeptides, cRGDfK and cRGDyK [72], where the amino group in the lysine residue can be easily conjugated by amide formation, and cRGDfC, where the thiol group on the cysteine gives Michael Addition with maleimide-functionalized moieties. Another largely employed ligand is the cyclic undecapeptide RGD4C, conformationally constrained by two disulfide bridges, that was identified in a phage display library [73]. Other integrin ligands comprise in their structure peptidomimetic portions (i.e. azabicycloalkanes, aminoprolines, diketopiperazines) able to confer the right spatial orientation to the RGD sequence for integrin binding and offering suitable group for functionalization [74-77].

The status of research on integrin-targeted drug delivery systems in cancer and other diseases, either by carriers with direct conjugation of an integrin ligand and a drug, or by different types of nanoparticles, loaded with appropriate drugs and functionalized with an integrin ligand on their surface, has widely been reviewed, underling different aspect of this issue [62, 67-71]. Here, we will focus our description to cytotoxic drugs conjugated to peptidic and peptidomimetic integrin ligands paying particular attention to molecules that have been protected by intellectual property. An overview of the reported molecules with the available biological data is presented in Table **2**.

Doxorubicin

The anthracycline antibiotic doxorubicin (DOXO), which inhibits the essential nuclear enzyme DNA toposoimerase II, that relaxes DNA supercoils to unable DNA replication, transcription and chromosome segregation, has been employed in the preparation of several integrin-drug-conjugates [78, 79]. A first example of doxorubicin conjugated to RGD-containing cyclic peptide targeting integrin $\alpha_v\beta_3$ was disclosed in 1998 by E. Ruoslahti and Pasqualini [73, 80]. The derivative, obtained by coupling the cytotoxic drug with the RGD-4C cyclopeptide, was used to treat mice bearing human MDA-MB-435 breast carcinoma at a sub-optimal dose of 30 μg of DOXO-equivalent every three weeks for 84 days. Animals treated with DOXO-RGD-4C conjugate showed improved tumor growth inhibition and a reduced number of metastases in comparison to animal treated with the free drug [73]. Moreover, the integrin-targeted drug resulted in less hepato- and cardiotoxicity than free DOXO as revealed by histopathological analysis. The authors ascribed these positive results to enhanced internalization by $\alpha_v\beta_3$ overexpressing MDA-MB-435 cell line, but experiments were not designed to clarify the mechanism of action of the conjugate.

Other research groups [81, 82] introduced enzymatically cleavable linkers in order to ensure preferred cleavage in cancer cells with respect to the stable amide linker. De Groot *et al.* conjugated the bicyclic RGD-4C to DOXO, introducing the tumor-specific D-Ala-Phe-Lys sequence, selectively recognized by the tumor-associated protease plasmin, which is involved in tumor invasion and metastasis **1** (Fig. **3**) [81]. Nevertheless, experiments on HT1080 fibrosarcoma cells as well as on HUVECs, designed to evaluate *in vitro* cytotoxicity, were not followed by *in vivo* studies.

Ryppa *et al*. compared integrin-targeted doxorubicin conjugates connected through either an amide bond or a proteinase cleavable peptide sequence [82]. The derivatives were obtained by reacting the thiolated divalent ligand E-[c(RGDfK)$_2$] with either a maleimido functionalized DOXO or with DOXO functionalized with a MMP2/MMP9-cleavable octapeptide **2** (Fig. **3**). The MMP-sensitive E-[c(RGDfK)$_2$]-DOXO conjugate was effectively cleaved in OVCAR-3 tumor homogenates, releasing DOXO. In contrast, no release of the anthracycline was observed for the E-[c(RGDfK)$_2$]-DOXO amide conjugate. Unfortunately, both

the constructs did not show relevant effects in mice xenografted with the human ovarian carcinoma OVCAR-3 cell line.

A further implementation of the MMP-cleavable derivatives was disclosed in a patent by KTB tumorschungsgesellschaft [83]. This patent claims prodrugs composed by at least one pharmaceutically and/or diagnostically active compound bound by a cleavable linker, a receptor targeting moiety and a protein-binding moiety which is capable of binding to a carrier molecule (such as albumin) aimed at improving lifespan in systemic circulation. One reported example involves the use of DOXO as the pharmaceutically active compound, cyclic E[c(RGDfK)]$_2$ peptide as the integrin receptor-targeting moiety, and a maleimido terminating linker, capable of binding *in situ* to a thiol-containing macromolecular carrier, as the protein-binding moiety. Nevertheless, the patent does not report data about the synthesis and biological activity of such a construct.

Paclitaxel

Paclitaxel (PTX) is an antimitotic agent which acts by stabilization of cell microtubules, thereby inhibiting cell division. Many examples involving integrin-targeted PTX prodrugs are based on conjugation at the 2'-hydroxy group. It is ascertained that irreversible derivatization of PTX at 2' position suppresses its cytotoxic activity [84]. For this reason, hydrolysable ester bounds are often preferred for conjugation [75, 85-88].

Chen *et al.* coupled the 2'-OH-group of PTX through a succinate spacer to the amino group of the E-[c(RGDyK)$_2$] divalent ligand **3** (Fig. **4**). The PTX-RGD conjugate inhibited cell proliferation with activity comparable to that observed for the free drug [85]. Nevertheless, although it showed higher tumor uptake and longer retention in comparison with the free drug, as observed by the biodistribution profile of ^{3}H-Labeled PTX-RGD in a metastatic breast cancer (MDA-MB-435) xenograft model, the conjugate was not able to inhibit tumor growth [86]. The stability of the conjugated construct was not evaluated, thus it is unknown if and when the free PTX is released during the time course of the assays and a premature release of the drug cannot be excluded [86].

A very similar derivative **4** (Fig. **4**) was synthesized by Ryppa *et al.* [87]. *In vitro* antiproliferation assays against HUVECs showed modest cytotoxicity only after short incubation times (30 min.) while effects similar to those observed with the free drug were reported after standard 72 h incubation. The short half-life of the PTX ester bond in the conjugate (~2 h at pH 7) leading to a premature release of the drug was proposed to explain the lack of selectivity in *in vitro* assays and as the cause of inefficacy observed *in vivo* in an ovarian carcinoma (OVCAR-3) xenograft model [87].

Manzoni *et al.* used a cyclic RGD peptidomimetic based on modified azabicycloalkanes (Aba) and Aminoproline (Ampro) scaffolds, disclosed for *in vitro* and *in vivo* $\alpha_v\beta_3/\alpha_v\beta_5$ integrin detection [74, 77, 89-94] for the delivery of PTX. The conjugates were obtained linking C2' PTX diglycolic or succinic esters with AbaRGD or AmproRGD integrin ligands through spacers of different length and hydrophilicity. All the nine synthesized compounds maintained their ability to bind integrin $\alpha_v\beta_3$ and showed remarkable cytotoxic activity in *in vitro* growth inhibition assays on a panel of $\alpha_v\beta_3/\alpha_v\beta_5$-over-expressing human tumor cell lines. One of the PTX conjugated prodrugs **5** (Fig. **4**) was evaluated *in vivo* in a platinum-resistant (IGROV-1/Pt1) ovarian carcinoma xenograft model, where enhanced activity in comparison with free PTX administered at twice the dose was found [88].

In a further study, the same research group investigated the potential of multipresentation in order to improve the binding affinity of the conjugates towards $\alpha_v\beta_3/\alpha_v\beta_5$ integrins. All the four synthesized compounds (three divalent and one tetravalent derivatives) presented enhanced binding affinity in comparison to the monovalent counterparts and showed remarkable cytotoxicity on a panel of human tumor cell lines. Among them, the bivalent derivative **6** (Fig. **4**) was selected for *in vivo* studies in ovarian carcinoma xenografts, showing an antitumor activity similar to that of PTX, but with a much more favorable toxicity profile [95].

More recently, Gennari and coworkers patented RGD-based integrin ligands based on diketopiperazine scaffolds (cyclo[DKP-RGD]) for conjugation with cytotoxic drugs [96]. In particular, examples of integrin ligands conjugated with PTX are reported. Among them, compound (cyclo[DKP-f3-RGD]-PTX), characterized by high affinity towards integrin $\alpha_v\beta_3$ and endowed with sufficient stability in both human and murine plasma, was tested *in vivo* using the platinum resistant ovarian carcinoma IGROV-1/Pt1 cells xenografted in immunodeficient mice, where decreased tumor growth with respect to free PTX was observed [75]. In a subsequent study, protease-sensitive dipeptides Val-Ala and Phe-Lys, connected to the PTX 2'-hydroxy group through a self eliminating p-aminobenzylcarbonate, were introduced to modulate the release of PTX [97]. The antiproliferative activities of two constructs **7** and **8** (Fig. **4**) with proteases cleavable dipeptides were compared with a compound conjugated through a stable amide link, *in vitro* on acute lymphoblastic leukemia cell line CCRF-CEM ($\alpha_v\beta_3$-) and its subclone CCRF-CEM$\alpha_v\beta_3$ ($\alpha_v\beta_3$+). Whereas the uncleavable conjugate resulted inactive in both cell lines, proteases cleavable derivatives showed improved antiproliferative activity in $\alpha_v\beta_3$ positive cells with a 7-fold enhanced effect with respect to free PTX [97].

Differently from the above reported examples, Brown and co-worker targeted integrin $\alpha_v\beta_6$. They conjugated 2'-maleimido-PTX to a tetramer bringing four copies of linear peptide H2009.1 (RGDLATLRQL) identified from a phage-displayed library as a high-affinity and specific ligand of integrin $\alpha_v\beta_6$ [98, 99]. The conjugate showed *in vitro* a weaker cytotoxic activity towards $\alpha_v\beta_6$-expressing non-small cell lung cancer H2009 cells than the free drug, but was more potent than the analogous conjugate displaying a scramble peptide sequence. *In vivo* experiments in a H2009 xenograft model revealed however comparable effects of the conjugate with the free PTX, although a delayed response was observed [99].

Camptothecin

Camptothecins act as inhibitors of DNA topoisomerase I, an enzyme which regulates multiple cell functions including

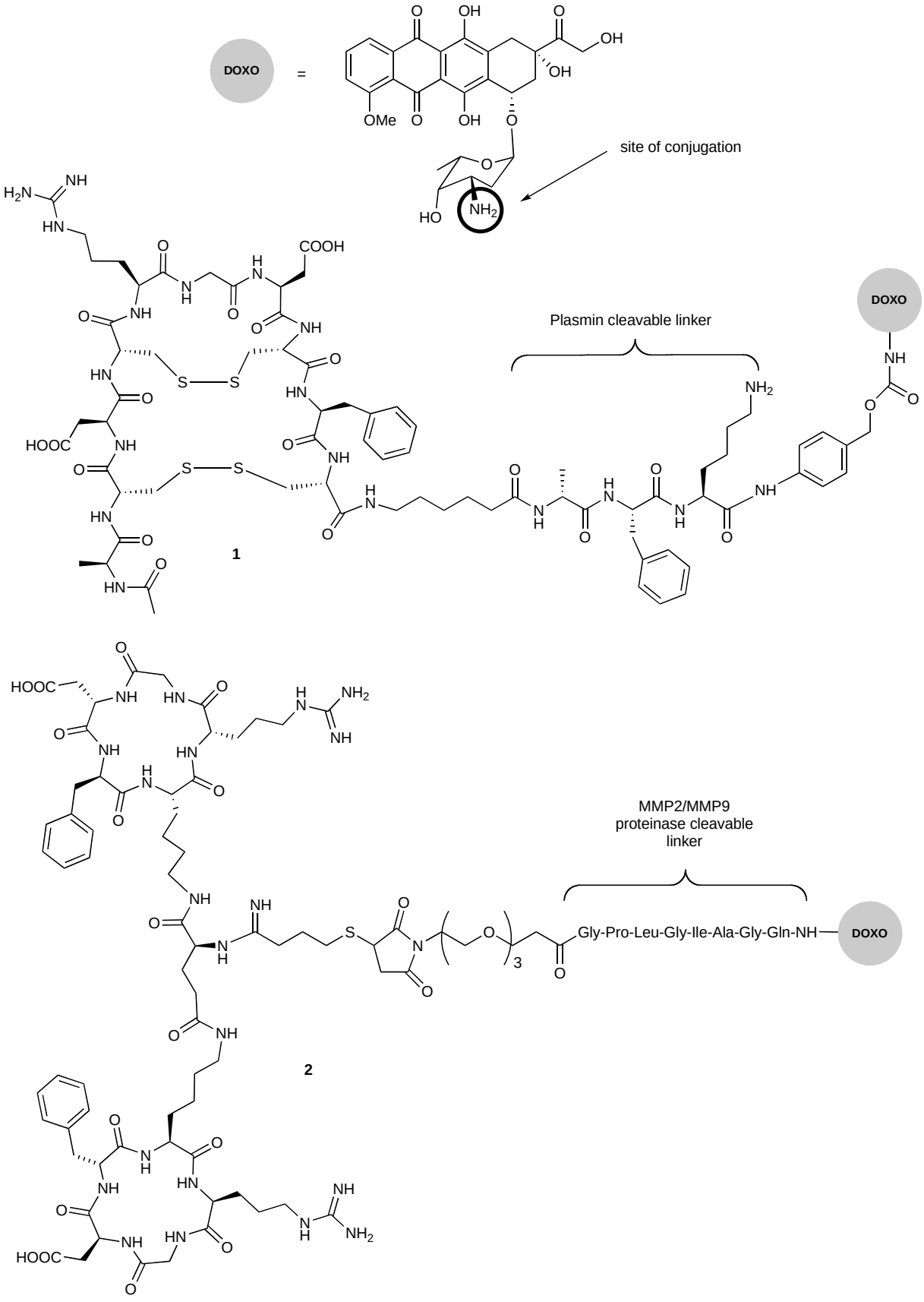

Fig. (3). Integrin-targeted doxorubicin conjugates. Chemical structures of conjugates between doxorubicin (DOXO) and integrin ligands through enzymatically cleavable linkers **1**, **2** [81, 82].

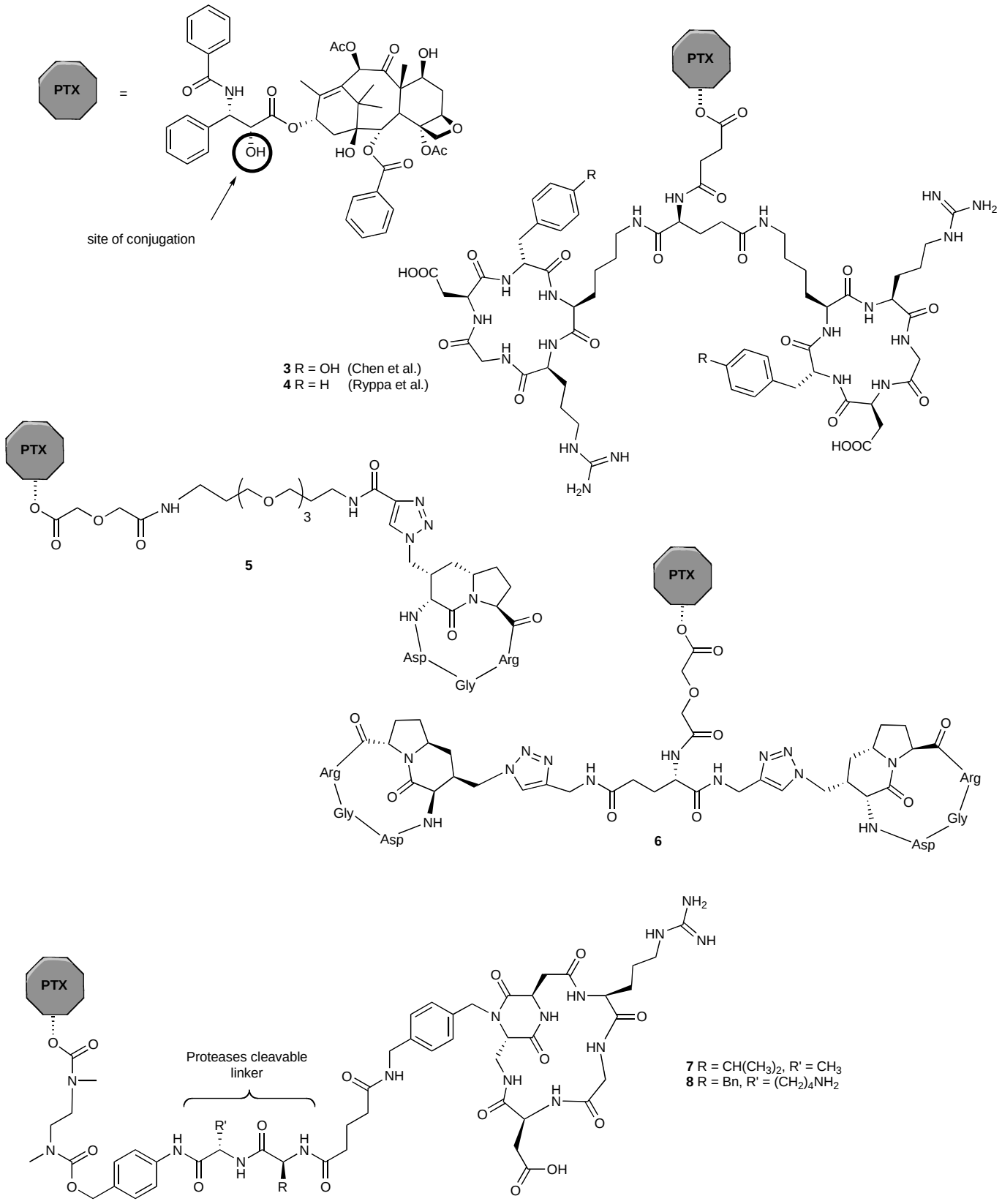

Fig. (4). Integrin targeted paclitaxel conjugates. Chemical structure of conjugates between integrin ligands and paclitaxel (PTX) linked at 2' OH through a succinic or a diglycolic linker (compounds **3-6**) [85-88, 95] or through proteases cleavable peptide linkers (compounds **7**,**8**) [97].

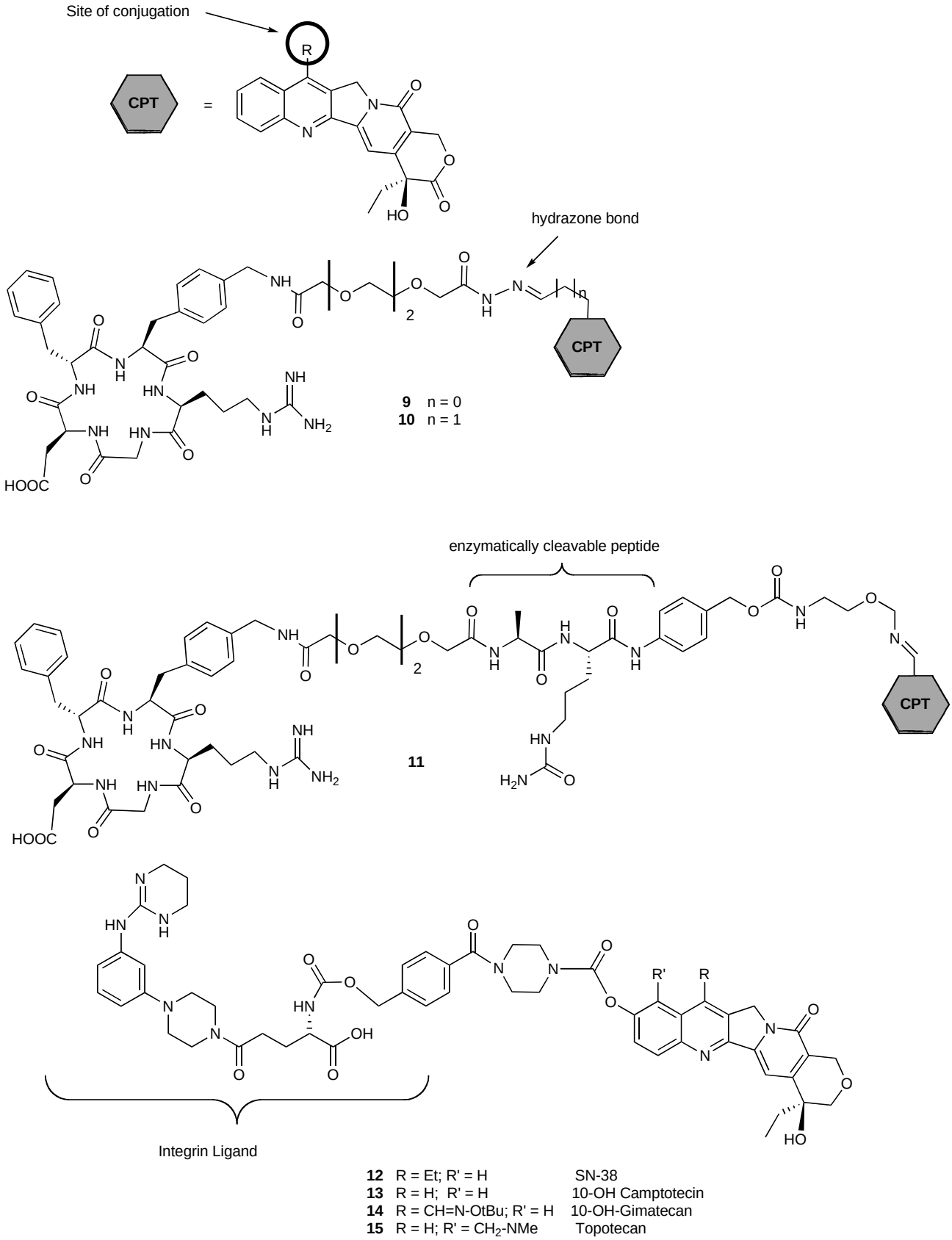

Fig. (5). Integrin targeted conjugates of camptothecins. Chemical structures of conjugates between integrin ligands and camptothecins functionalized at position 7 through pH-sensitive hydrazone bond **9**, **10** [102] or introducing an enzymatically cleavable linker **11** [104] and of 10-hydroxy-camptothecin derivatives **12-15** [107].

DNA replication, transcription and recombination [100]. Dal Pozzo *et al*. employed cycloRGD ligands equipped with functional groups suitable for conjugation [101] to prepare adducts with camptothecin (CPT) and its derivatives [102]. Synthesized compounds differed one from each other in the nature of the linker and of the chemical bond (stable amide or pH sensible hydrazone) between the drug and the ligand. Though all the conjugates maintained their affinity for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ isolated receptors, only compounds characterized by the hydrazone bond **9** and **10** (Fig. **5**) exhibited toxicity comparable with that of the parent drug in cells of prostatic (PC3), renal (A498) and ovarian (A2780) cancer. Similar conjugates between analogues of Cilengitide, opportunely modified with functional groups suitable for conjugation, and derivatives of CPT at position **7** (Fig. **5**), were patented by Sigma-Tau [103, 104]. The targeting and the cytotoxic units were connected through spacers designed to be stable in physiological conditions, but readily cleaved when the construct is internalized into cells. Their structure include an enzymatically cleavable peptide, and a short PEG linker whose length and conformation were designed to enhance solubility without altering binding affinity of the RGD ligand for integrins $\alpha_v\beta_3/\alpha_v\beta_5$. Binding affinity towards isolated $\alpha_v\beta_3/\alpha_v\beta_5$ receptors and cytotoxicity on human prostate and ovarian carcinoma were maintained for all the eight synthesized conjugates. *In vivo* evaluation of derivative **11** (Fig. **5**), delivered at 25 mg/Kg subcutaneously to mice xenografted with the A2780 human ovarian carcinoma cells, revealed a potent antitumor effect showing complete regression of all tumors at the maximum tolerate dose of 25 mg/Kg, persistence of the effect after the last treatment and good tolerability [103].

Alloatti *et al*. conjugated SN-38 (10-hydroxy-7-ethylcamptothecin), an active metabolite of irinotecan [105], and other 10-hydroxy-camptothecin derivatives to a non-peptidic RGD mimetic first synthesized by Iwama *et al*. [106], opportunely modified to allow conjugation [107]. The four novel synthesized compounds **12-15** (Fig. **5**) were assessed for cytotoxicity *in vitro* on human ovarian carcinoma cells overexpressing integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, as well as on human prostate cancer cells with low integrins expression, showing for the conjugate between SN-38 and RGD mimic a better antiproliferative activity on the ovarian cancer cells. This latter conjugate showed improved stability in plasma and comparable results in terms of plasmatic life span and tumor growth inhibition in PC3 prostate cancer and A498 renal carcinoma xenograft mouse models in comparison with the free irinotecan.

Gilad *et al*. conjugated cRGDfK with the hydroxyl group of CPT using a carbamate bond [108, 109]. The compound **16** (Fig. **6**) was tested *in vitro* on a panel of tumor cell lines, in comparison with the free drug and with CPT conjugated with a linear peptidic sequence containing both the RGD integrin binding motif and the CD13 targeting moiety NGR. While free CPT showed non-specific cytotoxicity towards all the tested cell lines (H1299, PC-3 and HEK), the CPT-cRGDfK conjugate **16** resulted active towards $\alpha_v\beta_3$ expressing H1299 cells and PC-3 cells (in this latter case showing enhanced toxicity in comparison with free CPT), but not towards $\alpha_v\beta_3$ negative HEK cells. Finally, CPT conjugated with the linear RGD-NGR containing peptide preserved moderate activity only toward integrin expressing cell lines [108].

In order to improve the activity and/or reduce the risk of developing cell resistance, the same authors synthesized conjugates between cRGDfK modified at the side chain of its Lys residue with a sixth amino acid containing two functional sites which enabled the loading either of two therapeutic equivalents of an anticancer drug or of two different drugs (CPT and/or chlorambucil, CLB) [110]. Different conjugation strategies (ester, amide or carbamate) were adopted to bind the drugs to the integrin ligand to obtain different releasing profiles.

The four double loaded conjugates **17-20** (Fig. **6**) were tested in a cell growth inhibition assay against human non-small cell lung carcinoma H-1299 cells and murine melanoma B16F10 cells over-expressing $\alpha_v\beta_3$ integrin and human embryonic kidney (HEK) 293-β1 cells with low $\alpha_v\beta_3$ expression. All the double loaded conjugates resulted more active in comparison both with their monovalent counterparts and with the free drugs towards $\alpha_v\beta_3$ integrin expressing cancer cell lines. Interestingly, it appears that the enhanced toxicity is not simply due to the doubled drug content, as demonstrated by the fact that mono-conjugate compounds at double dose still showed lower grown inhibition values. This aspect is of particular relevance in an attempt to reduce the side effects derived from potent drugs administration.

Finally, a theranostic agent comprising CPT conjugated through a disulphide cleavable linker with a naphthalimide moiety (as a fluorescent reporter) to cRGDyK cyclic peptide (as integrin targeting unit) was developed by Lee *et al*. **21** (Fig. **6**) [111]. Confocal microscopy was employed to assess the preferential uptake of the construct by $\alpha_v\beta_3$ overexpressing U87 human glioma cells over integrin low expressing C6 rat glioma cells. Integrin-mediated endocytosis of the construct was further confirmed by blocking experiments in the presence of the endocytosis inhibitor okadaic acid. CPT release, after cleavage of the disulfide bond by GSH followed by intramolecular cyclization and cleavage of the neighboring carbamate bond, was further detected by red-shifted fluorescence emission at 535 nm (off-on signal) from naphthalimide moiety. This approach also allowed determining, by using a known endoplasmic reticulum-selective dye, that the dissociation takes place at the endoplasmic reticulum (ER) whereas non targeted construct was released in mitochondria. Viability data in U87 cell line showed a superior cytotoxicity for the integrin targeted derivative relative to the untargeted counterpart.

Platinum(IV) Prodrugs

Platinum compounds are widely used in antitumor therapy and cisplatin is the component of various standard chemotherapy regimens [112]. Cisplatin conjugates are usually obtained through the synthesis of Pt(IV) complexes. These complexes have the double advantage of being less toxic to liver and kidney than cisplatin itself, while preserving the potency on cancer cells and offering the possibility of conjugation to other molecules. In facts, the high chemical stability of Pt(IV) prodrugs can favor their transport across the body avoiding side reactions with proteins and other

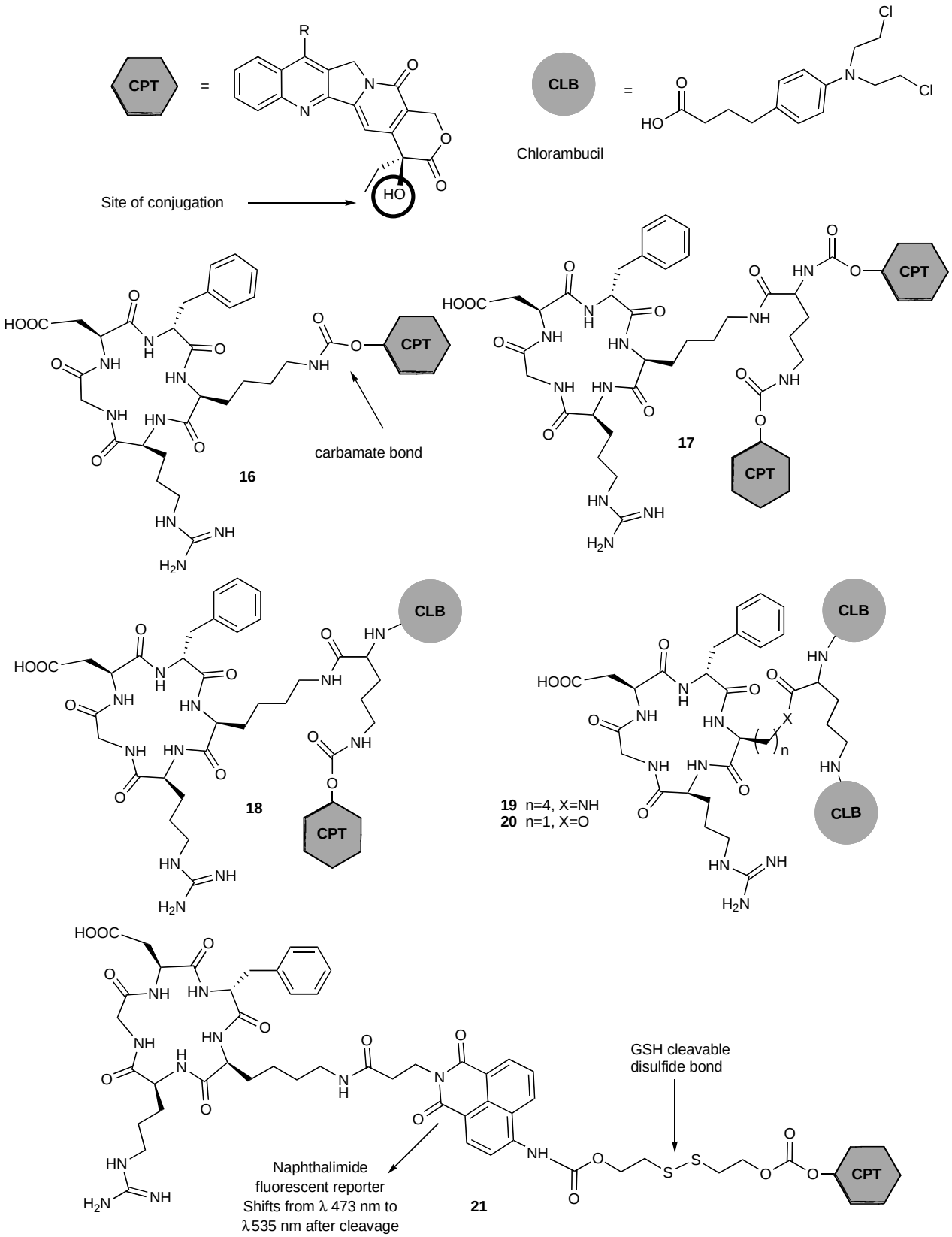

Fig. (6). Integrin targeted camptothecin obtained by conjugation at the hydroxyl group. Chemical structures of conjugates between integrin ligand and camptothecin (CPT) and/or chlorambucil **16-20** [108-110]. Theranostic CPT-derivative conjugated through a reducible disulfide bond **21** [111].

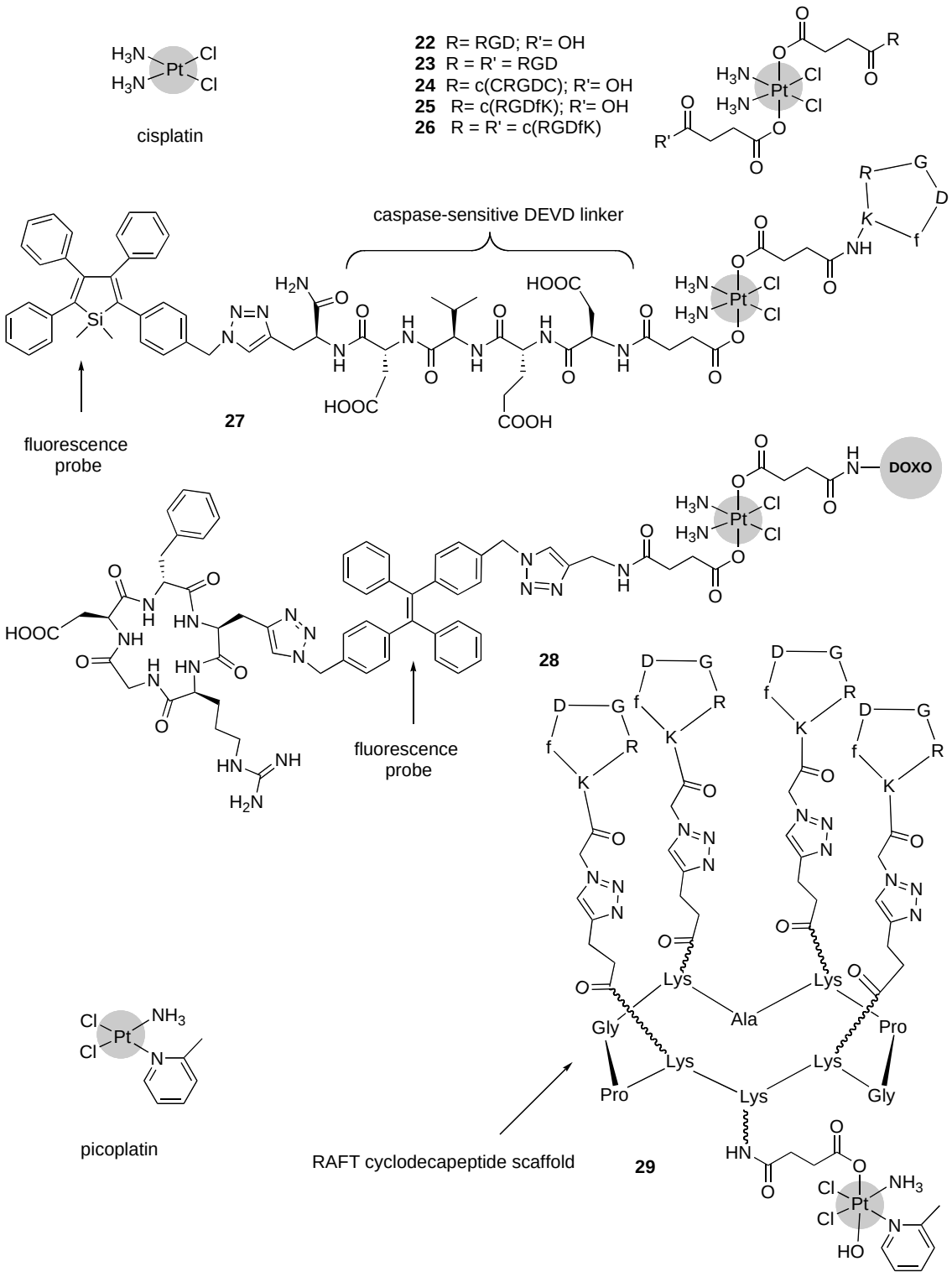

Fig. (7). Integrin targeted Pt(IV) prodrugs. Chemical structures of conjugates between Pt(IV) complexes and integrin ligands **22-26** [115]. Theranostic Pt(IV)-prodrugs **27**, **28** [117, 118]. Multivalent Pt(IV)-prodrug **29** [120].

biomolecule allowing a higher amount of complex to reach the tumor site, where it can be intracellularly activated by reduction to Pt(II) species [113]. Moreover, functional groups on the axial ligands in Pt(IV) complexes provide the sites for conjugation of prodrug with targeting ligands as disclosed by Lippard *et al.* [114] who reported some examples of integrin-targeted complexes where the axial succinates of *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(succinate)_2$] were mono- or bis -conjugated by amide linking to linear RGD. Derivatives comprising cyclic c(CRGDC) and c(RGDfK) integrin ligands were also reported in a further study **22-26** (Fig. **5**) [115]. These constructs were able to inhibit cell proliferation in endothelial (HUVEC) and human cancer cell lines *in vitro*.

An integrin $\alpha_v\beta_3$ targeted Pt(IV) prodrug with theranostic potential has been recently described [116, 117]. The construct **27** (Fig. **7**) was synthesized by linking cRGDfK and caspase-sensitive DEVD peptide (previously connected by click chemistry to the azidomethyl-tetraphenylsilole fluorophore) to the N-hydroxysuccinimide-activated *cis,cis,trans*-diammine-dichloro-disuccinatoplatinum(IV). DEVD cleavage would induce aggregation and activation of the fluorophore, thus providing an apoptosis sensor. The envisaged sequence involves $\alpha_v\beta_3$ targeting and internalization, followed by intracellular reduction of Pt(IV) and release of active Pt(II) that would induce caspase-3 mediated apoptosis, revealed by the activatable fluorophore. *In vitro* experiments were performed in $\alpha_v\beta_3$ integrin rich U87MG human glioblastoma cells, and in MCF-7 breast cancer and 293T cells, characterized by low integrin expression. At confluence the construct was added at final concentration of 5 μM. Confocal microscopy showed that the fluorescence of U87MG cells increased gradually with cellular apoptosis, reaching a maximum at 6 h, whereas only weak fluorescence was observed in the control cells. Moreover, the fluorescence of the U87MG was almost suppressed by pre-treating cells with either the free integrin $\alpha_v\beta_3$ ligand cRGDfK or the potent caspase-3 inhibitor 5-[(*S*)-(+)-2-(methoxymethyl)pyrrolidino]sulfonylisatin. Cytotoxicity testing paralleled the fluorescence results. Co-localization experiments with anti-active caspase-3 antibody also confirm good overlap of the activatable fluorophore and immunofluorescence. The results were so considered as an example of real-time, non-invasive theranostic indicator, although limited to experiments on cells *in vitro* [117].

In a subsequent study, the research group adopted the same strategy for the construction of a theranostics delivery system containing both Pt(IV) prodrugs and doxorubicin [118]. The construct is composed by a targeting cRGD moiety linked to a tetraphenylene (TPE) activatable fluorophore and conjugated to *cis,cis,trans*-diammine-dichloro-disuccinatoplatinum(IV). The other succinic group of Pt(IV) prodrug is conjugated trough an amide bond with DOXO **28** (Fig. **7**). In this design, TPE fluorescence is quenched due to energy transfer to DOXO while the resultant fluorescence of DOXO can serve for prodrug tracking. Confocal microscopy demonstrated that the construct was preferentially accumulated by $\alpha_v\beta_3$ rich MDA-MB-231 cells in comparison with MCF-7 breast cancer cells and 293T cells (human embryonic kidneys transformed with large T antigen) both characterized by low $\alpha_v\beta_3$ expression. The release of active Pt(II) as well as of DOXO anticancer drugs upon reduction following internalization in MDA-MB-231 cells is witnessed by time-dependent enhancement of fluorescence intensity from cleaved cRGD-TPE, while the red fluorescence of DOXO can be seen first in the cytoplasm (after 1 h incubation) and after longer incubation time (4 h) in the cell nucleus. Cell viability assay in MDA-MB-231 showed that cRGD-TPE-Pt-DOXO construct displayed higher cytotoxicity in comparison to the treatment with either cisplatin or DOXO.

A tetrameric c(RGDfK) construct **29** (Fig. **7**), obtained employing a regioselectively-addressable functionalized template (RAFT) cyclodecapeptide scaffold [119], was employed in order to enhance the activity and selectivity of the *cis*-ammine(2-methylpyridine)-dichloridoplatinum(II) (picoplatin). Mono and tetrameric integrin-targeted drug delivery systems were obtained by conjugation of the picoplatin Pt(IV) prodrug carrying a succinic axial group [120]. The anti-proliferative activity of the tetrameric construct, in comparison with the mono-derivatized conjugate and the free drug, was assessed in $\alpha_v\beta_3/\alpha_v\beta_5$ integrin expressing melanoma SK-MEL-28 cells, and in CAPAN-1 and 1BR3G cell lines, both with low integrin expression. The tetrameric conjugate showed enhanced antiproliferative activity in SK-MEL-28 cells with respect to picoplatin, whereas no cytotoxicity was observed in control cells lacking $\alpha_v\beta_3$ and $\alpha_v\beta_3$ integrins. Efficacy was found to correlate with intracellular accumulation of platinum (determined by ICP-MS), which increased proportionally with integrin expression levels and number of conjugated ligands. Interestingly, the unconjugated tetrameric RAFT-RGD peptide also showed an anti-proliferative activity in the melanoma cancer cells, thus indicating the need of further studies to determine the role of multivalent RGD ligand [120].

CURRENT AND FUTURE DEVELOPMENTS

Although the biological role of integrins has been studied for several decades and novel molecular details of their functions are still being discovered, the clinical use of peptide- and peptidomimetic drugs in antitumor therapy has been only partially satisfactory. At the preclinical level, the promising features of this strategy have emerged because peptide- and peptidomimetic drug conjugates are expected to avoid the main mechanisms of acquired resistance that are known to limit the efficacy of most of the chemotherapeutic agents [121-123]. In principle, for those conjugates which are endowed with reasonable stability which allows to reach the targetable tumor cells, mechanisms of resistance may involve drug conjugate-target interactions as well as cell response of tumor cells to the conjugate. Thus, alterations preventing the action of the conjugated cytotoxic agent, such as defects in the DNA repair (for instance in the case of conjugates with DNA damaging agents) or alterations in susceptibility to drug-induced cell death, as well as alterations preventing the interaction of the peptide- and peptidomimetic drug conjugates with integrin may underlie drug resistance. In the latter case, down-regulation of integrin expression may account for lack of activity, because target-dependent uptake of the drug conjugate occurs together with target-independent accumulation processes. Mutations of genes coding for integrin subunits may alter ligand specificity or result in truncated integrin versions, possibly affecting binding of peptide or peptidomimetics. Besides expression of the targeted integrin by the tumor and drug-conjugate internalization, the efficiency of the release of the free drug by deconjugation - also dependent on linker features (sensitivity to pH, reduction or cleavability by proteases) and lysosomal degradation of the conjugates - may account for tumor cell sensitivity or resistance to treatment.

The complexity of the integrin-mediated signaling involving multiple adhesome proteins (e.g. FAK, and scaffolding proteins) and known not to be limited to the plasma membrane, but implicating different subcellular compartments such as endosomes or the nuclei as well as extracellular vesicles – specifically exosomes – suggests the need to better exploit integrins to treat cancer.

Table 2. Biological Data Available for Integrin Targeted Drug Conjugates.

Drug	$\alpha_v\beta_3$ Ligand	Cleavage Type	N°	Stability Data Available	$\alpha_v\beta_3$ Binding IC_{50}	*In vitro* Cytotoxicity	*In vivo* Model	Patent	Ref.
DOXO	RGD-4C			No			MDA-MB-435 in mice	WO1998/010795	[73, 80]
	RGD-4C	Enzymatic	1	Yes	25 nM[b]	HT1080 HUVEC			[81]
	E-[c(RGDfK)$_2$]	Enzymatic	2	Yes		HUVEC	OVCAR-3 in mice		[82]
	E-[c(RGDfK)$_2$]	Enzymatic		No				WO2008098788	[83]
PTX	E-[c(RGDyK)$_2$]	Hydrolysis	3	No	25.9 nM[c]	MDA-MB-435	MDA-MB-435 in mice		[85, 86]
	E-[c(RGDfK)$_2$]	Hydrolysis	4	Yes		HUVEC	OVCAR-3 in mice		[87]
	cAba-RGD	Hydrolysis	5	No	220 nM[d]	IGROV-1 IGROV-1/Pt1 H460 U2-OS	IGROV-1/Pt1 in mice		[88]
	cAba-RGD	Hydrolysis	6	No	35 nM[d]	IGROV-1 IGROV-1/Pt1 H460 U2-OS	IGROV-1/Pt1 in mice		[95]
	c-DKP-RGD	Hydrolysis		Yes	5.2 nM[d]	IGROV-1 IGROV-1/Pt1 U2-OS SKOV3 PANC-1 MIA-PaCa2	IGROV-1/Pt1 in mice	WO2013114180	[75, 96]
	c-DKP-RGD	Enzymatic	7, 8	Yes	7: 13.3 nM[d] 8: 52.4 nM[d]	CCRF-CEM ($\alpha_v\beta_3$-) CCRF-CEM subclone ($\alpha_v\beta_3$+)			[97]
	linear peptide H2009.1[a]	Hydrolysis		Yes		H2009 H460	H2009 in mice		[99]
CPT and analogues	RGD cyclopen-tapeptide	Hydrolysis	9, 10	Yes	9: 11.66 nM[e] 10: 6.01 nM[e]	PC3 A498 A2780	A2780 in mice		[102]
	RGD cyclopen-tapeptide	Enzymatic	11	Yes	30.40 nM[e]	PC3 A2780	PC3 and A2780 in mice	WO2009141240	[103, 104]

Table (2) contd….

Drug	$\alpha_v\beta_3$ Ligand	Cleavage Type	N°	Stability Data Available	$\alpha_v\beta_3$ Binding IC_{50}	*In vitro* Cytotoxicity	*In vivo* Model	Patent	Ref.
	Peptidomimetic	Hydrolysis	12-15	Yes	12: 1.3 nM[e] 13: 2.28 nM[e] 14: 4.83 nM[e] 15: 4.97nM[e]	PC3 A2780	A498 in mice		[105]
	cRGDfK	Hydrolysis	16	Yes		PC3 H1299 HEK-293 B16F10			[108, 109]
	cRGDfK	Hydrolysis	17, 18	Yes		H-1299 B16F10 HEK-293			[110]
	cRGDyK	Reduction	21	Yes		U87			[111]
Pt(IV) prodrugs	Linear RGD, c(CRGDC) and c(RGDfK)	Reduction	22-26	No		HUVEC BCE HMVEC-d U87 ASPC1 MES-SA HeLa		US 7138520	[114, 115]
	cRGDfK	Enzymatic;Reduction	27	Yes		U87MG MCF-7 293T		WO2015112092	[116, 117]
	cRGDfK	Reduction	28	Yes		MDA-MB-231 MCF-7 293T			[118]
	tetrameric c(RGDfK)	Reduction	29	No		SK-MEL-28, CAPAN-1 1BR3G			[120]

[a] RGDLATLRQL peptide targeting integrin $\alpha_v\beta_6$

[b] Inhibition of the binding of ^{125}I-RGD-modified protein to $\alpha_v\beta_3/\alpha_v\beta_5$ on H5V cells

[c] Inhibition of the binding of ^{125}I-echistatin to MDA-MB-435 cells

[d] Inhibition of the binding of biotinylated vitronectin to isolated $\alpha_v\beta_3$ integrin

[e] Inhibition of the binding of ^{125}I-echistatin to isolated $\alpha_v\beta_3$ integrin

Abbreviations: Aba: azabicycloalkane; DKP: Diketopiperazine

Cell lines: 1BR3G: fibroblasts; 293T: Human embryonic kidney cells; A2780: Human ovarian carcinoma; A498: Renal carcinoma; ASPC1: Human pancreatic adenocarcinoma cells; B16F10: murine melanoma cells; BCE: Bovine capillary endothelial cells; CAPAN-1: Pancreatic cancer cells; CAPAN-1: pancreatic cancer cells; CCRF-CEM: Acute lynphoblastic leukemia cells; H1299:human non-small cell lung carcinoma cells; H2009 :Human lung cancer cell lines; H460: Human large cell lung carcinoma; H460: Human lung cancer cell lines; HEK-293 :Human Embryonic Kidney 293-(β1); HeLa: cervical sarcoma; HMVEC-d: Human dermal microvascular endothelial cells; MCF-7: breast cancer cells; HT1080:fibrosarcoma cells; HUVEC: Human Umbilical Vein Endothelial Cells; IGROV-1/Pt1: Cisplatin resistant human ovarian adenocarcinoma; IGROV-1: Human ovarian adeno-carcinoma; MCF-7: Breast cancer cells; MDA-MB-231: human breast adenocarcinoma; MDA-MB-435: Human breast carcinoma; MES-SA: uterine sarcoma; MIA-PaCa2: human pancreatic carcinoma; OVCAR-3; Human ovarian carcinoma cells; PANC-1: Human pancreatic carcinoma; PC3: prostatic carcinoma ; SK-MEL-28: Human melanoma; SKOV3: Human ovarian carcinoma cell line; U2-OS: Human osteosarcoma; U87: Human glioma cells; U87MG: Human glioblastoma

In this regards, heterogeneity of integrin expression pattern should be better investigated in an attempt to identify personalized antitumor treatments to selectively target metastatic disease. Indeed, the integrin expression pattern has been predicted to be modulated within primary tumor and metastases at different sites [34]. Moreover, integrin expression has been shown to evolve throughout progression [124].

A historical view on development of antitumor agents [78, 125] indicate the multiple strategies that have been engaged to bypass the limited efficacy of antitumor therapy, including gene therapy [126], drug combinations and experimental studies against cancer stem-like cells (e.g. PIN-1) [127] supporting the need to widen the approaches to attack the tumor cells. In this context, the development of integrin-targeted peptide- or peptidomimetic-drug conjugates has been proposed as promising [75, 88, 95]. In fact, excellent results have been obtained in preclinical studies in which the possibility to use such conjugates to target drugs to tumor cells has appeared to be advantageous allowing to reduce the dose of chemotherapeutic agents administrated to achieve a therapeutic effect. Nevertheless, though almost 15 years of research elapsed from the seminal paper by Arap *et al* [73], none of the proposed integrin targeted-drug conjugate constructs entered in clinical trials. This picture, though not encouraging, has forced researchers to point their efforts on finding innovative approaches to the problem. The reasons why such targeted drugs have not entered clinical trials so far may be multiple and may include problems related to the stability of the conjugates, including unexpected poor accumulation in experimental *in vivo* models or premature deconjugation before interaction with the integrins expressed by tumor cells. Thus, it has not been possible to evaluate side effects resulting from toxicity towards healthy cells. In fact, the analysis of cytotoxicity on normal cell lines *in vitro* suffers from major bias related to the fact that such cells proliferate less than cancer cells and are therefore less susceptible to the conjugated drugs.

Research efforts are expected to contribute to further clarify the biological role of integrins. In this regard, the recognition of the multiple roles of integrins in cooperation with other components of the tumor microenvironment or associated with cell structures e.g. heparan sulfate proteoglycans is expected to improve our understanding of cell communication mechanisms, such as those occurring through extracellular vesicles [28].

The recently described function of integrins may allow a better interpretation of the biology of these receptors in view of future clinical use. In this context, exosomal integrins may be helpful to predict organ-specific metastasis [22] and the use of exosome proteomics may be exploited in finding biomarker for selecting those patients that may benefit from treatment with peptide or peptidomimetic-drug conjugates. In addition, the interference of integrin-targeted therapies with sites beyond the plasma membrane (e.g., endosomes, nucleolus) is only in part clear, but could impact on clinical outcome [128]. This is the case for example of interference with ICD, a type of cell death that stimulates host antitumor immune response and can be triggered in cancer cells by radiotherapy or treatment with certain antitumor agents [129-131].

The development of peptide- and peptidomimetic-drug conjugates may benefit from the newly acquired knowledge generated from biological studies. In fact, new potent and selective integrin ligands can be synthesized on the basis of these new findings. On the other hand, the deeper understanding of integrin signaling has led to the design of dual action compounds targeting both integrins and other tumor associated pathways (i.e. VEGFR mediated angiogenesis or caspases dependent apoptotic pathway) [132-134].

Moreover, given the variety of integrin receptors which are capable of recognizing RGD-motif in natural ligands, (namely $\alpha_{IIb}\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_5\beta_1$, $\alpha_8\beta_1$) a continuous critical analysis of the principle of selectivity has been pursued by several research groups with the aim of finding selective ligands able to target specific tumor types, thereby opening the way to personalized medicine [135].

Finally, a widespread approach to enhance the efficacy of integrin-targeted drugs is based on the employment of nanosized carriers. The use on integrin targeted nanodelivery system has been the focus of a huge amount of studies, as supported by the number of reviews devoted to the subject [62, 67-69, 71]. This approach exploits nanoparticles in order to enhance the content of the transported drug to the tumor site, but also to take advantage of nanoparticles specific chemical physical properties to improve tumor uptake in a synergistic way both based on active targeting and passive accumulation through the well-known enhanced permeability and retention effect.

LIST OF ABBREVIATIONS

Akt	=	v-Akt Murine Thymoma Viral Oncolgene Homolog
AP2	=	Adaptor Protein Complex 2
b-FGF	=	Basic Fibroblast Growth Factor
CPT	=	Camptothecin
Csk	=	c-Src Tyrosine Kinase
DAB2	=	Disabled Hoomolog 2
DOXO	=	Doxorubicin
ECM	=	Extracellular Matrix
EMT	=	Epithelial-Mesenchymal Transition
ER	=	Endoplasmic Reticulum
ERK1/2	=	Extracellular Signal-Regulated Kinase 1/2
FAK	=	Fokal Adhesion Kinase
FLIP	=	Filamin A Interacting Protein
GSK 3β	=	Glycogen Synthase Kinase-3 Beta
ICAM	=	Intercellular Adhesion Molecule
ICD	=	Immunogenic Cell Death
IMD	=	Integrin-Mediated Death
JNK	=	Jun N- Terminal Kinase
MAPK	=	Mitogen-Activated Protein Kinase
MMP	=	Matrix Metalloproteinase

NF-kB = Nuclear Factor k-B

PDGF = Platelet-Derived Growth Factor

PI3K = Phosphatidylinositol 3-Kinase

PTX = Paclitaxel

RAFT = Regioselectively-Addressable Functionalized Template

RGD = Arginine-Glycine-Aspartate

Src = Rous Sarcoma Oncogene Cellular Homolog

STAT1 = Signal Transducer and Activator of Transcription 1

STAT6 = Signal Transducer and Activator of Transcription 6

TGF-β = Transforming Growth Factor β

VCAM = Vascular Cell Adhesion Molecule

VEGF = Vascular Epithelial Growth Factor

CONFLICT OF INTEREST

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

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