2. INTRODUCTION

2.1 Integrins

2.1.1. Integrins biology

Integrins are heterodimeric transmembrane proteins belonging to a family of cell adhesion receptors evolutionary old and playing pivotal roles in physiological and pathological processes, such as neo-angiogenesis, cancer spreading and osteoporosis. The combination of 18 α-subunits and 8 β-subunits forms at least 24 αβ heterodimers. These structures depend upon the presence of divalent cations (calcium and magnesium) and are finally composed by an extracellular domain, a single transmembrane region and a short (30-40 amino acids circa) cytoplasmic tail (**Figure 1**). The cytoplasmic domain deserves a particular attention, because it is crucial for the regulation of integrin activity and function. It controls the integrin affinity state and its ECM ligand-binding activity, but it also promotes cellular responses upon extracellular ligand binding [4].

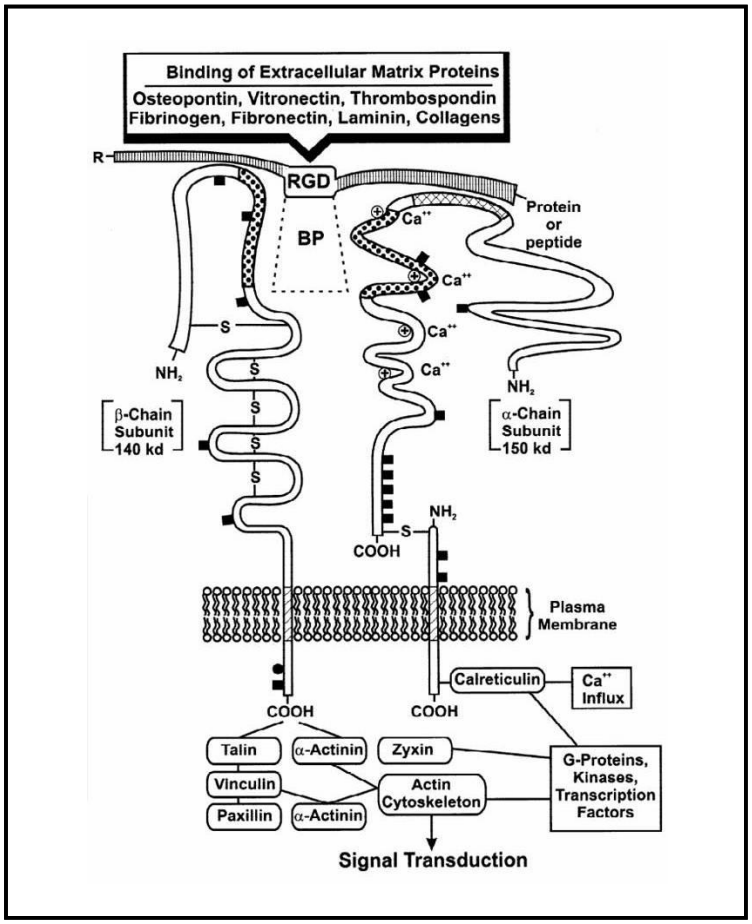

Figure 1. *How a universal integrin receptor heterocomplex looks like [40].*

The cell usually displays an excess of β subunits, so that the amount of the α subunit determines the amount of the heterodimeric receptor going on the cell surface. Then, the α subunit is the limiting factor in the formation of the heterodimer [5]. Crystallization studies underlined that inactive integrins exist in a compact

bent conformation. Integrins are activated upon binding with their extracellular ligands (inside-out signalling), thus changing in this way their molecular conformation according to individual variations. This leads to the so-called outside-in signalling, which activates complex and cell-specific signalling events depending on the other molecular partners involved. As will be underlined below, these signalling events are mainly related to cell migration, proliferation, differentiation and survival.

Integrins are activated through the binding with their extracellular ligands. Activated integrins cluster with few signalling and adaptor proteins from the cytoplasm, including growth factor receptors, cytokine receptors and trafficking molecules. In fact, integrins lack of intrinsic kinase activity and need to recruit and activate kinases such as FAK and SFK. This clustering gives rise to dynamic and macromolecular protein structures globally termed adhesion complexes, able to ensure the mechanical link between the inner side of the cell and the ECM. If localized at the cell periphery these are known as focal contacts (tiny complexes) or focal adhesions (large complexes). If localized along stress fibers they are called fibrillar adhesions [1].

Integrins act as molecular bridges between the ECM and the cytoskeleton, thus displaying mechanical functions and affecting cell biology by means of complex intracellular signalling pathways. It is noteworthy that mechanical tension itself is able to promote and increase integrin affinity [5]. Integrins are also able to mediate cell adhesion to immune cells. The ability of integrins to mediate adhesion between different cell types or between a single cell and the ECM is strictly dependent upon specific short peptide sequences recognized by integrins on their ligands. Some integrins recognize only a single ligand, whereas others can bind several ligands (**Table I**) [40]. To this regards, integrins play pivotal roles in traction for cell motility and invasion, in remodeling of ECM by means of localization of proteases, in cell growth through adhesion-dependent control of proliferation, in cell signalling thanks to the cross-talk with growth factors and cytokine receptors.

Integrin subunits	Extra-cellular matrix Protein/Cell Ligand	Recognition site (AA)*	RGD sensitive (+) (−)	Presence‡ / Absence I-Domain +	Functional activities
$\alpha_1\beta_1$	LAM, Col	YIGSR, RHDS	−	+	Reduced expression in breast carcinoma
$\alpha_2\beta_1$	Col, PLT, LAM, ENC, cell-to-cell	DGEA, RHDS	−	+	Inhibits malignancy of mammary carcinoma
$\alpha_3\beta_1$	FBN, Col, INV, EPL, $\alpha_2\beta_1$, $\alpha_3\beta_1$, LAM, KAL, ENT	RGD	+	−	Expressed in most tumor cells
$\alpha_4\beta_1$	FBN, VCAM-1 PP-HEV, CSF	EILDV, IDAPS, REDV	−	−	Inhibits metastases in melanoma and β-lymphoma
$\alpha_5\beta_1$	FBN, INV, PLT	LDV, RGD, PHSRN	+	−	Overexpression suppresses growth and tumorigenesis
$\alpha_6\beta_1$	LAM, INV, PLT, EPL	VIGSR?	−	−	Elevated in breast, liver, and NSCL carcinoma
$\alpha_7\beta_1$	LAM	VGVAPG, YIGSR	−	−	Increased expression in melanomas
$\alpha_8\beta_1$	BAL, TEN	IDG, LDV, IDA	−	−	Basement membrane-associated
$\alpha_9\beta_1$	MER, TEN	IDG, LDV, IDA	−	−	Expressed in colon carcinoma
$\alpha_v\beta_1$	FBN, VTN	RGD	NR	−	Increased in anaplastic tumors
$\alpha_1\beta_2$	$ICAM_{1,2,3}$	KELLLPGNNRKV	−	+	Leukocyte to endothelial cell adhesion
$\alpha_m\beta_2$	C3bi, FBN, FAX, ICAM	KQAGDV	−	+	Adhesion, phagocytosis, complement binding
$\alpha_x\beta_2$	C3bi, FBN	GPRP	−	+	Leukocyte adhesion, complement binding
$\alpha_{11b}\beta_{IIIa}$	FBN, FBG, FIB, VWF, VTN, TSP	KQAGDV, RGD, HHLGGAKQAGDV, RYD	+	+	Required for activated platelet aggregation
$\alpha_v\beta_3$	BSP, FIB, VTN, FBN, FBG, PLC, αCol, PBP, VWF, TSP, OSP, Col	RGD, RYD	+	−	Essential for angiogensis for melanoma and angiogenic cells
$\alpha_6\beta_4$	LAM, KAL, MER	VGVAPG? YIGSR?	?	−	Correlates with malignancy in keratinocytes
$\alpha_v\beta_5$	VTN, FBN, PBP, TAT	RGD	+	−	Suppresses anchorage independence
$\alpha_v\beta_6$	FBN	RGD	+	−	Virus-associated fusion
$\alpha_4\beta_7$	FBN, VCAM, MADCAM	EILDV	NR	NR	Endothelial and mucosal lining-associated actions
$\alpha_v\beta_8$	NR	NR	NR	NR	Reproductive/development activities
$\alpha_7\beta_9$	NR	NR	NR	NR	NR
$\alpha_pS2\beta_p$	FBN, BMP	β-subunit	NR	NR	ECM interaction during Drosophila development

Table I. *Members of the integrin supergene family categorized according to their ECM ligands, amino acid recognition sites, and function (adapted from [40]).*

Integrins are considered the main receptors for ECM proteins (vitronectin, fibronectin, laminins, collagens). Moreover, integrins can bind also immunoglobulin-like cell surface molecules such as VCAM-1 or ICAM-1, both involved during immune cells rolling in inflammatory settings. Integrins are characterized by redundancy, so that the same ligand may be bound by different integrins. This provides a great advantage in particular conditions such as wound healing or tissue remodeling, when the cellular response is more important than the nature of the ECM proteins involved. This promiscuity accords to the cellular need to have the availability of different signalling events and pathways from the same ECM proteins. Finally, integrins may also directly bind a number of growth factors including VEGF or FGF. This evidence, together with the known cross-talk between integrins and growth factors receptors, suggests the importance of integrin proteins not only in cell-matrix binding, but also in the cell-microenvironment context.

As underlined before, integrin ligation promotes signalling events leading to cell spreading, migration, survival and proliferation. Many signalling pathways activated by integrins are also activated by growth factor receptors, so the concomitant engagement of integrins and growth factor receptors optimizes the global yield of the activation through signals integration. The four major pathways activated by integrins and relevant to this work are Ras-MAPK, PI3K-Akt, NF-kB and Rho-family GTPases. In particular, Ras, PI3K and NF-kB pathways are mainly involved in cell migration, proliferation and survival. On the other side, the pathway of Rho-family GTPases is basically related to integrin recycling from the membrane (**Figure 2**).

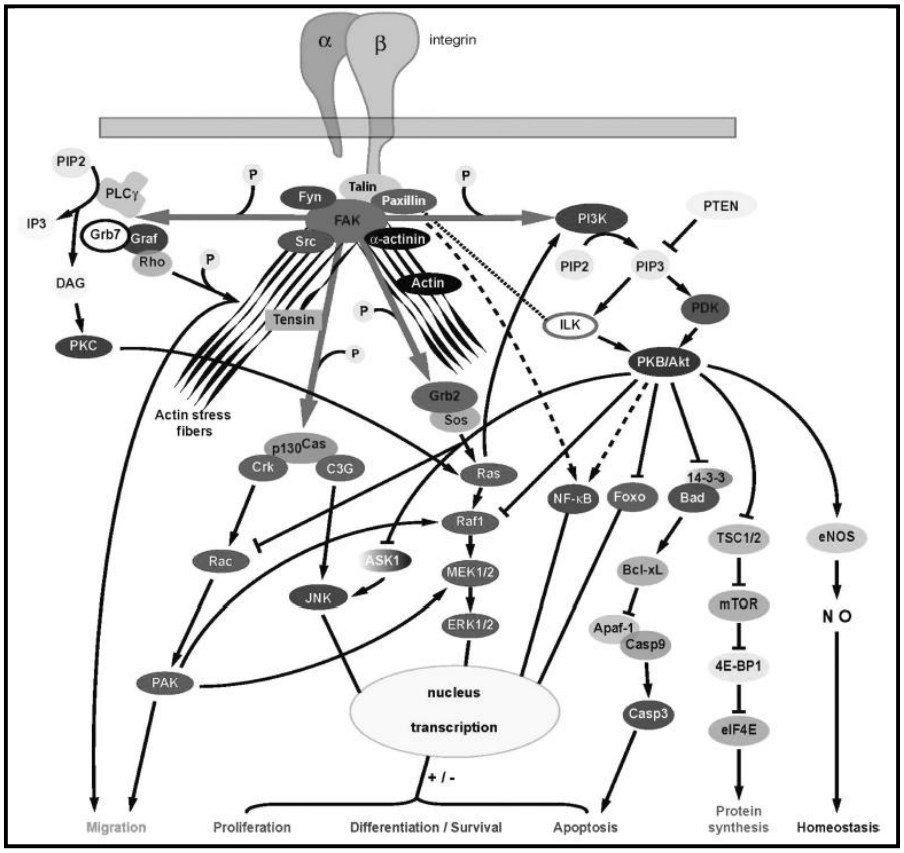

Figure 2. *Signaling pathways initiated by integrins at focal contacts [1].*

Integrins, in particular some β1 and αv integrins, take advantage of FAK or the Src-family kinases to stimulate the ERK kinase. Moreover, integrin αvβ3 ligation enhances MAPK activation induced by VEGF or FGF-2.

Integrins are able to activate Akt kinase by means of FAK, PI3K or ILK activation. The activation of Akt by VEGF, angiopoietin-1 or insulin is strictly dependent upon integrin-mediated adhesion. Akt is generally involved in promoting cell proliferation, migration and survival, thus impairing pro-apoptotic molecules such as Bax and Bad.

The pathway of small GTPases is crucial in integrin biology because Rho, Rac and cdc42 regulate the organization of actin cytoskeleton and modulate cell proliferation. In particular, Rho GTPase is actively involved in integrin trafficking and recycling at the leading edge of the cell, thus playing a pivotal role in regulating directional cell migration.

Finally, ligation of integrin αvβ3 promotes the activation of NF-kB and promotes cell survival by inducing the expression of anti-apoptotic molecules (such as Bcl-2) and the down-regulation of pro-apoptotic genes (such as p53 and p21) [1].

2.1.2 Integrins in angiogenesis and lymphangiogenesis

2.1.2.1 Introductory overview on angiogenesis and lymphangiogenesis

Cells require oxygen and nutrients for their survival. Considering that the diffusion limit for oxygen is within 100 and 200 μm, cells growing beyond this size absolutely need to recruit new blood vessels. Angiogenesis is the development of new blood vessels from pre-existing ones, and starts with an initial vasodilation of pre-existing vessels. This phenomenon is generally accompanied by an increase in vascular permeability and a degradation of the surrounding matrix, because ECM is enriched in chemotactic and pro-angiogenic factors (e.g. VEGF). This setting ultimately promotes endothelial cell migration toward the anatomical sites where there is a major need of oxygen and nutrients. A complex vascular network finally forms, lined by endothelial cells ready to function under a variety of conditions [12, 16].

Angiogenesis is crucial during human development and adult life because it is able to promote embryonic development, tissue repair and fertility. In particular, embryonic vessels are assembled from endothelial precursors, so that this primitive network may expand by sprouting or intussusception. However, angiogenesis is also one of the most important clinical manifestations of pathological conditions, such as chronic inflammation, tumor growth and tumor metastasis. Tumor vessels develop by sprouting or intussusception as well, but also circulating endothelial precursors – shed from the vascular wall or mobilized by bone marrow – may also contribute to tumor angiogenesis. Moreover, tumor cells are able to surround an existing or a growing vessel in order to form a perivascular cuff, thus rendering firmer the developing vasculature. When tumor sizes enlarges and the inner zones of the tumor are far from the vasculature, this covering ultimately leads to the delivery of oxygen and nutrients to the necrotic center of the tumor [19].

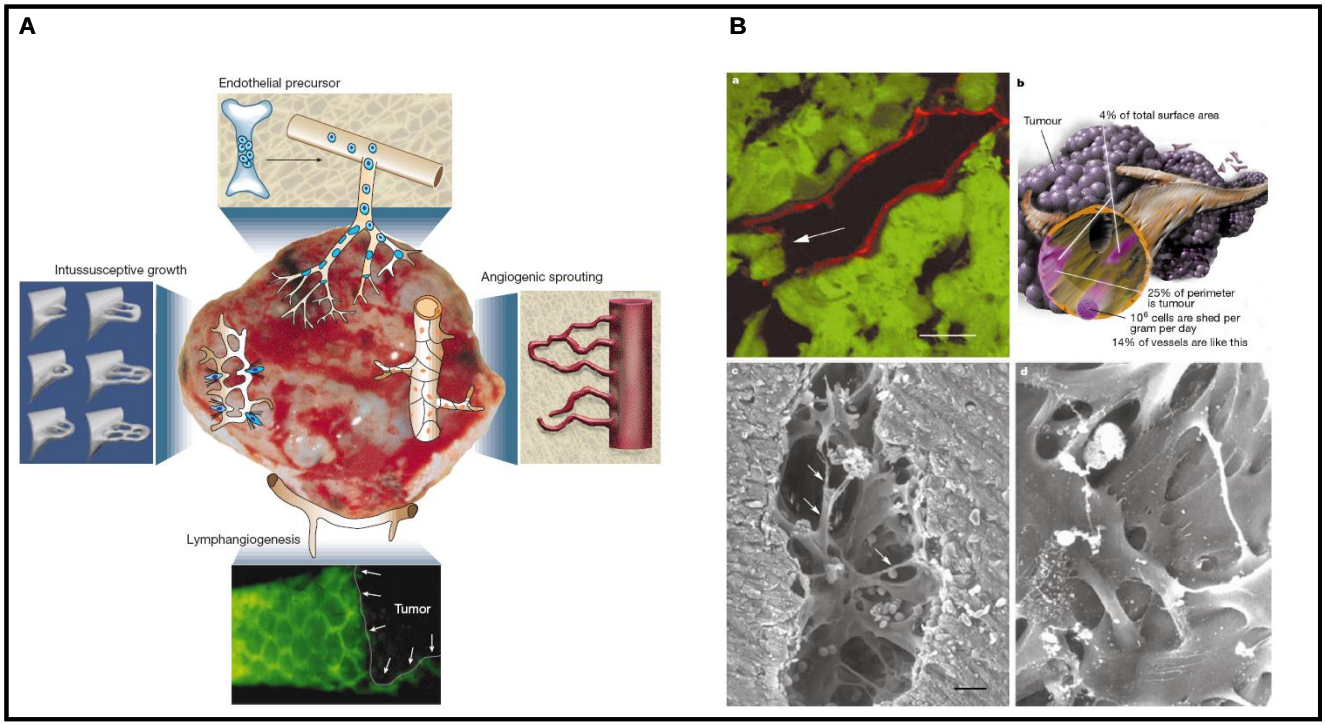

Figure 3. *General mechanisms of angiogenesis and lymphangiogenesis.* ***A:*** *Cellular mechanisms of tumor (lymph) angiogenesis. Tumor vessels may grow by: angiogenesis; intussusception; vasculogenesis; lymphangiogenesis (providing a gate for metastases)* ***B:*** *Chaotic and mosaic vessels in tumors. (a) Cancer cell in the lining of a tumor vessel (green: tumor cells; red: tumor endothelial cells); (b) quantification of mosaic vessels; (c) abnormal endothelial cells within a tumor; (d) multiple intercellular openings. Adapted from [12].*

In addition to tumor cells, macrophages and fibroblasts may produce or stimulate tumor cells to release pro-angiogenic factors, cytokines and ECM-degrading proteases, so when the net balance is in favor of angiogenesis the so-called 'angiogenic switch' is activated. In this way, both tumor and host cells participate in tumor neo-angiogenesis and, finally, in its growth and metastatization [47].

Tumor vessels are generally chaotic, structurally and functionally abnormal. Tumor vasculature is not well organized and vessels are tortuous and dilated, with excessive branching and shunts. Because there are a lot of endothelial *fenestrae* and transcellular holes, tumor vessels are leaky and highly permeable. This leads to an increase of interstitial fluids and finally facilitates lymphatic metastases, which are strictly dependent by tumor-driven lymphangiogenesis as well (**Figure 3**) [12].

Lymphangiogenesis is the development of new lymphatic vessels (**Figure 4**). Lymphatic system is composed by a network of blind-ended, thin-walled lymphatic capillaries collecting vessels and specialized secondary immune organs. The role of lymphatic vessels is to drain protein-rich interstitial fluids and immune cells through the lymph nodes, collecting fluids back to the circulation. Indeed, lymph nodes are typically the first organ to display tumor metastasis, and sentinel lymph node monitoring is the elective method used to detect metastases. Then, lymphatic system has a pivotal role in cancer biology too. Tumor cells and TAMs may induce the formation of new vascular and lymphatic vessels, so that cell trafficking and tumor spreading are highly facilitated [48].

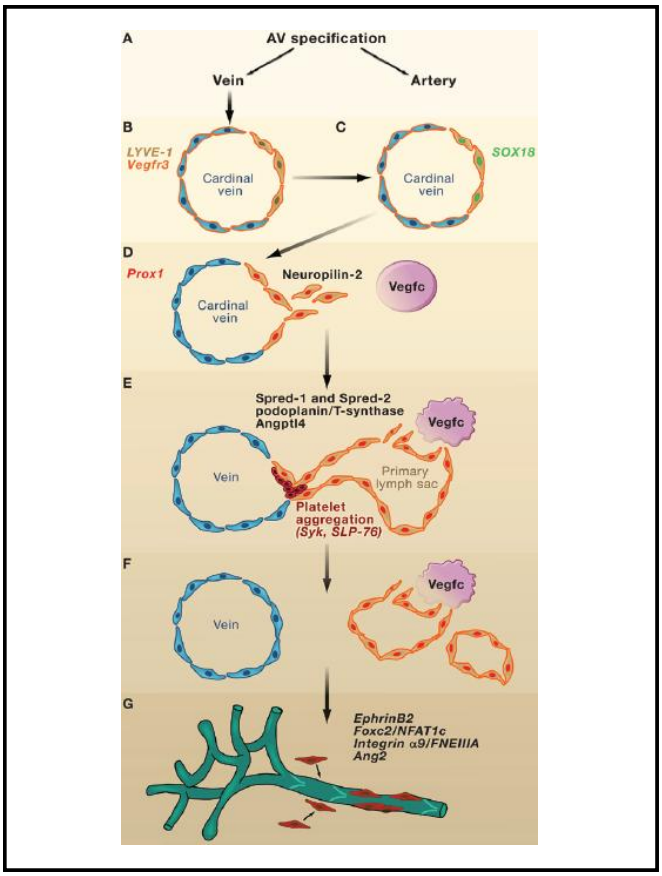

Figure 4. *Development of the mammalian lymphatic vasculature (Adapted from [48]).*

Few data confirm that some endothelial integrins are very important in the regulation of cell growth, survival and migration during angiogenesis and lymphangiogenesis. In particular, pro-angiogenic stimuli and growth factors are able to up-regulate integrins αvβ1, α2β1, α4β1, α5β1 and αvβ3. Moreover, integrin αvβ5 is constitutively active on endothelial cells, but functionally activated only upon VEGFR-2 mediated signalling.

2.1.2.2 The αv integrins

The integrins αv have key roles in embryonic development of blood vessels in placenta and brain. Integrin αvβ3 is widely expressed on blood vessels in human tumor biopsies but not on vessels in normal tissues. This seems to suggest that integrin αvβ3 is not required for vascular development, although it has been demonstrated that its inhibition and antagonism may promote vascular disruption and endothelial cell apoptosis, respectively. In addition, TAMs are able to stimulate integrin αvβ3 expression by enhancing the secretion of bFGF, IL-8 and TNF-α by tumor cells. The important role of integrin αvβ3 in angiogenesis is given by the different role exerted by αv and β3 subunits [10]. On the whole and as will be underlined below, the loss of integrin αvβ3 may be compensated by an up-regulation of other integrin-related signalling pathways [27]. To this regards, integrin αvβ5 has an angiogenic pathway distinct from that of integrin αvβ3. Integrin αvβ5 is up-regulated by TNF-α and VEGF and is involved in vascular permeability, highlighting its possible role in tumor metastasis. Integrin αvβ5 is fundamental for the survival of developing vessels, although it is not required for vascular development. Moreover, integrin αvβ5 exerts a compensatory effect

by means of a cross-talk with VEGFR-2 in β3 null mice [44]. Finally, integrin αvβ8 is required for proper brain blood vessels development, and probably also in the onset of blood-brain barrier [38].

2.1.2.3 Fibronectin-binding integrins

Fibronectin-binding integrins are essential for developmental angiogenesis. Fibronectin is a key ECM protein deposited by endothelial cells during normal or tumor angiogenesis, and may be bound by integrins α5β1, αvβ3 and αvβ5 (on RGD sequence) or by integrins α4β1 and α9β1 (on other domains). From this binding snapshot it appears that β1 integrins are crucial for developmental angiogenesis. Given that *Itga5* null embryos are lethal, it is clear that integrin α5β1 has a pivotal role in vasculogenesis and angiogenesis [33]. This is particularly relevant for this work. Integrin α5β1 is poorly expressed on quiescent endothelial cells, but it is up-regulated by pro-angiogenic molecules such as IL-8. Integrin α5β1 promotes endothelial cells survival by PKA blockade, and its antagonists have been demonstrated to impair tumor growth thus increasing tumor regression. Finally, although it has been demonstrated that integrin α5β1 is expressed by a sub-population of lymphatic vessels, it has no part in tumor lymphangiogenesis [22].

Integrins α4β1 and α9β1 are two other fibronectin binding integrins. Integrin α4β1 is expressed by endothelial cells and pericytes, binds VCAM-1 and mediates the extravasation of lymphocytes. Antagonists of integrin α4β1 have been demonstrated to promote cell death in both endothelial cells and pericytes, by interfering with VEGF (pericytes to endothelium cross-talk) and PDGF (endothelium to pericyte cross-talk) pathways [21]. Moreover, it has been reported that the inhibition of α4β1 by means of natalizumab (Tysabri) treatment promoted the onset of PML in multiple sclerosis patients [32]. This is an important observation, given that integrins on bone-marrow derived cells promote angiogenesis and the homing of myeloid and endothelial progenitors.

Integrin α9β1 is the most recent fibronectin-binding integrin demonstrated to have a role in angiogenesis, but this molecule is important during lymphatic system development as well. Mice null for this integrin are lethal, and it is important for the onset of a fully functional lymphatic system. In addition, integrin α9β1 binds several ECM proteins and directly binds VEGF-A, so that blocking antibodies are able to suppress VEGF-A induced angiogenesis [37, 50].

2.1.2.4 Laminin-binding integrins

Laminin-binding integrins are α1β1, α2β1, α6β1 and α6β4. It has been demonstrated that integrins α1β1 and α2β1 are not required for angiogenesis in the embryo, but literature displays a lot of confusion about their functions. Integrins α6β1 and α6β4 are not required for endothelial cells proliferation, but they are necessary for cell adhesion and migration. In particular, capillary endothelial cells *in vivo* express high levels of integrin α6β1 and antibodies directed against this receptor have been demonstrated to prevent endothelial cells tube formation *in vitro*. In addition, the treatment of endothelial cells with siRNAs against integrin α6β1 may inhibit their migratory activity [4].

2.1.2.5 Integrins in lymphangiogenesis

Specific lymphatic markers have been already identified, such as the homeobox transcription factor PROX1, the VEGFR-3, the mucoprotein podoplanin and LYVE1 [47]. However, evidence is arising that integrins may have a role in lymphangiogenesis too.

The observation that mice lacking integrin α9β1 display post-natal lethality due to chylothorax (accumulation of lymph in the pleural cavity) points out that integrin α9β1 is necessary for the development of a fully functional lymphatic system [29]. Moreover, PROX1 is able to upregulate integrin α9β1 and VEGFR3 expression *in vivo* [39]. In addition, integrin α9β1 is able to directly bind VEGF-C and VEGF-D, thus promoting lymphatic endothelial cells migration [50].

It has been then demonstrated that VEGF-A is able to drive tissue repair-associated lymphatic vessel formation through VEGFR-2 and integrins α1β1 and α2β1 [28].

Finally, integrin α5β1 is expressed by a subset of lymphatic endothelial cells in the inflamed cornea but plays no role in tumor lymphangiogenesis [20], whereas integrin α4β1 is highly expressed by tumor lymphatic endothelium [22]. In both cases, antagonists of integrin α5β1 or α4β1 are able to block inflammatory lymphangiogenesis and tumor metastasis [4].

2.1.3 Integrins in cancer

2.1.3.1 Integrins regulate cell survival and apoptosis

It has been demonstrated that a number of integrins is highly expressed in human metastatic cancers. In fact, integrins are able to promote cancer cells migration, survival and invasion because of their ability to degrade basement membrane by their interaction with proteolytic enzymes (e.g. metalloproteases). In addition, it is noteworthy that integrins have the ability to either enhance cell survival or initiate apoptosis depending on environmental cues. Epithelial cells generally need to be linked one another and to the surrounding tissues, otherwise their physiology is impaired. Thus, the binding between epithelial cells and the surrounding tissues promotes cell maintenance, whereas epithelial cells detachment is the main cause of cell death. Then, ligated integrins drive cell survival, whereas unligated integrins promote cell apoptosis by means of anoikis and IMD. Anoikis is a form of programmed cell death induced by cell detachment from the surrounding ECM. IMD occurs in response to the accumulation and apparent clustering of unligated or antagonized integrins on the cell surface, which promotes the apoptotic cascade driven by caspase-8. (**Figure 5**). The loss of caspase-8 by means of integrin αvβ3-mediated Src activation is a way commonly used by tumor cells to avoid and acquire resistance to IMD, thus becoming highly invasive and gaining a metastatic phenotype [19].

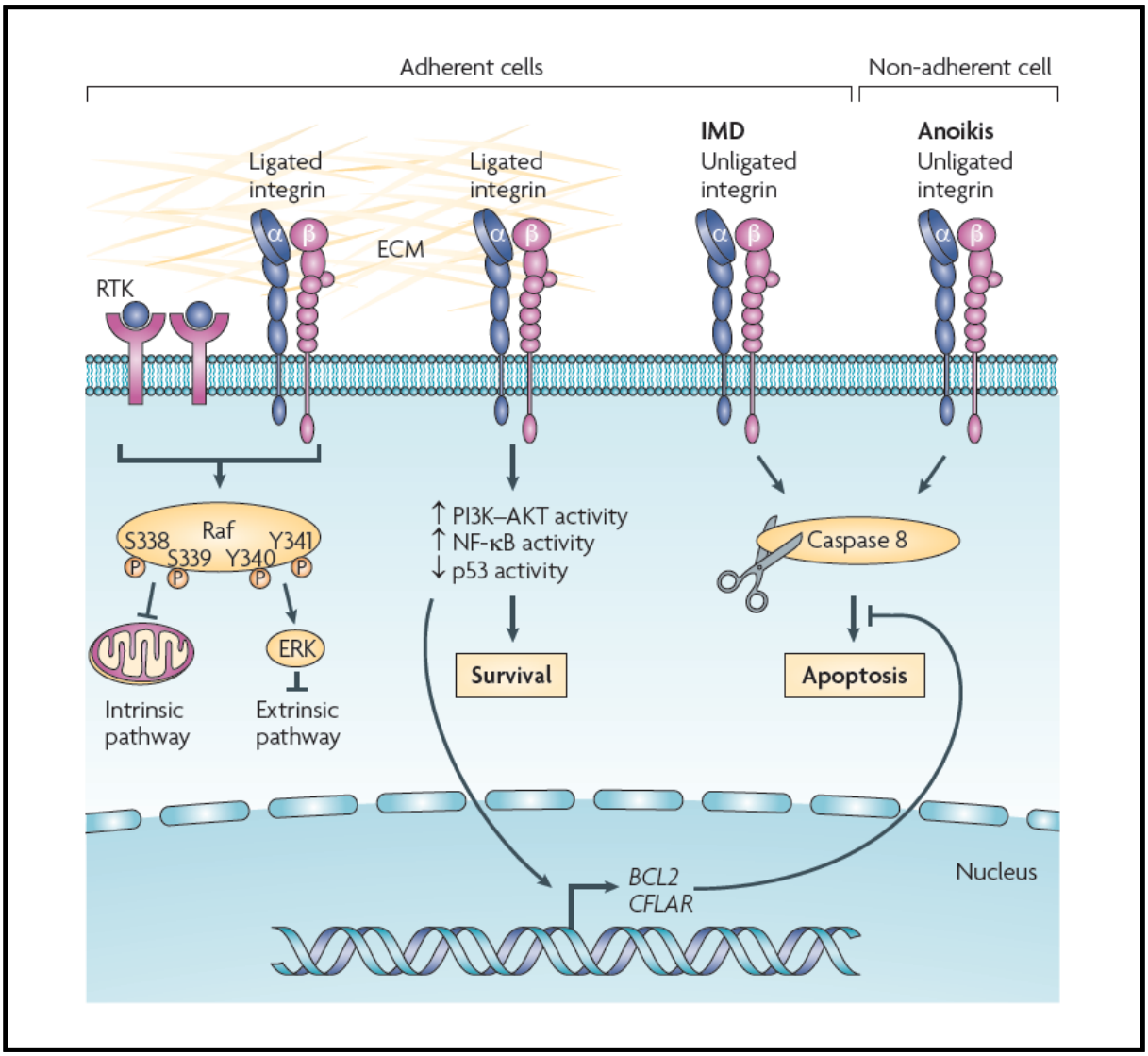

Fig. 5. *Integrin-mediated survival versus apoptotic pathways [19].*

The major integrins expressed by tumor cells are integrins αxβ1 and αvβ3. On the whole, these molecules are able to drive cancer cell invasion, proliferation, survival and also the EMT (through the activation of TGF-β by integrins αvβ6, αvβ8 and αvβ3). The extent to which integrins contribute to cancer progression strictly depends upon the cell type (**Table II**). Integrins α6β4, α6β1, αvβ5, α1β1, and α3β1 are normally expressed by epithelial cells but may contribute to tumor migration, proliferation and survival. Integrins αvβ3, αvβ6 and α5β1 are highly up-regulated in some tumors but are expressed in low amounts in adult epithelia. Indeed, integrin α2β1 deserves a particular attention, as will be highlighted below.

Tumour type	Integrins expressed	Associated phenotypes
Melanoma	αvβ3 and α5β1	Vertical growth phase [35,172–174] and lymph node metastasis[173,175]
Breast	α6β4 and αvβ3	Increased tumour size and grade[176], and decreased survival[177] (α6β4). Increased bone metastasis[36–38,64] (αvβ3)
Prostate	αvβ3	Increased bone metastasis[39]
Pancreatic	αvβ3	Lymph node metastasis[40]
Ovarian	α4β1 and αvβ3	Increased peritoneal metastasis[178] (α4β1) and tumour proliferation[179] (αvβ3)
Cervical	αvβ3 and αvβ6	Decreased patient survival[41,180]
Glioblastoma	αvβ3 and αvβ5	Both are expressed at the tumour–normal tissue margin and have a possible role in invasion[181]
Non-small-cell lung carcinoma	α5β1	Decreased survival in patients with lymph node-negative tumours[182]
Colon	αvβ6	Reduced patient survival[109]

Table II. *Integrins in cancer progression [19].*

Integrin α6β4 promotes tumor growth and invasion through the activation of ERK and PI3K/Src pathways, respectively. Given that the β4 subunit may directly promote cancer cell migration, invasion and escape from apoptosis, and the over-expression of integrin α6β4 correlates with a poor prognosis in various human cancers (e.g. breast cancer). In addition, the co-expression of integrin α6β4 with Net1 (a guanine-nucleotide exchange factor of Ras) is a marker for aggressive disease in node-positive, breast cancer patients [23].

Integrin α6β1 is a laminin-binding receptor involved in regulating tumor cell invasion. It has been demonstrated that this integrin may promote pancreatic cells interactions with fibroblasts during tumor progression [26].

Integrin α3β1 is another laminin-binding integrin able to regulate the expression of MMP-9, thus promoting tumor invasion and progression in particular during the vertical growth phase of melanoma. Moreover, in hepatomas and in mammary tumor metastases integrin α3β1-paxillin complex activates FAK pathway and promotes cells proliferation and survival [43].

Integrins α1β1 and α2β1 play a pivotal role in some tumors invasion (e.g. melanoma) and are up-regulated in most carcinoma-derived cells. However, it has been demonstrated that the expression of integrin α2β1 may also decrease in tumor cells, potentially correlating with an increase in tumor cell dissemination. These evidences seem to suggest that integrin α2β1 might also function as a tumor suppressor [19, 43].

Integrins αvβ3 and αvβ5 play important roles in promoting cancer cell biology as well. Integrin αvβ3 cross-talks with VEGFR in melanoma, glioblastoma and angiogenic endothelial cells. In addition, it is expressed in solid cancers characterized by lymph-node metastases, thus suggesting its importance in driving metastatic tumor progression. Integrin αvβ3 is able to bind and activate MMP-9 or uPAR and to mediate the adhesion of malignant cells to platelets (heterotypic interaction with integrin αIIbβ3) or to vascular endothelial cells (homotypic interaction with vascular integrin αvβ3). In addition, integrin αvβ3 mediates cancer cell migration

and invasion in osteosarcoma through the activation of MEK, ERK and NF-kB pathways or Src and PKC pathways, respectively. There are some evidences that integrin αvβ3 might also induce cell death in glioblastoma and melanoma because of the IMD process [17, 31]. Integrin αvβ5 is generally over-expressed in malignant tumor cells, angiogenic endothelial cells within the tumor and metastatic breast cancer cells. In addition, the cross-talk between integrin αvβ5 and EGFR has an important role in pancreatic cancer, as will be discussed below.

The fibronectin-binding integrin α5β1 may bind and activate MMP-1, thus promoting solid tumor invasiveness (e.g. prostate cancer). A ternary complex formed by integrin α5β1/EFGR/uPAR is correlated with poor prognosis and strongly enhances tumor migration and invasion. Moreover, the interaction of such a complex with Angiopoietin-1 is able to drive metastases to the lymph-nodes.

The role of integrin αvβ6 in cancer cell biology has been already clearly established. This molecule is up-regulated by VEGF in gastric carcinoma-associated endothelial cells and can be considered a prognostic biomarker linked to a poor survival. Integrin αvβ6 is minimally expressed by normal adult tissues, but it is present in high amounts in some cancer cells (e.g. pancreatic, cervical, lung and colon cancer cells). In this regard, the observation that colon cancer-derived metastatic liver cells express higher amounts of integrin αvβ6 in respect of primary colon cancer cells seem to suggest that this molecule could have remarkable pro-metastatic functions. In addition, integrin αvβ6 may block the intrinsic apoptotic pathway in colon cancer cells.

Finally, Src and ERK activation by integrin α9β1 promotes cell migration and the up-regulation of integrin α9β1 enhances the production of NO through of the activation of inducible iNOS. In fact, the lack of the activation of iNOS or Src impairs integrin α9β1-dependent migration [43].

2.1.3.2 Integrins cross-talk with growth factors, cytokines and their receptors

Integrins are able to cross-talk with growth factors, cytokines and their receptors. This cooperative signalling plays a pivotal role in the regulation of tumor cell adhesion, migration, invasion, survival and in the homeostasis of angiogenic endothelium. In addition, integrins and growth factors or cytokine receptors may reciprocally regulate the surface expression of one another and the release of the respective ligands (**Figure 6**).

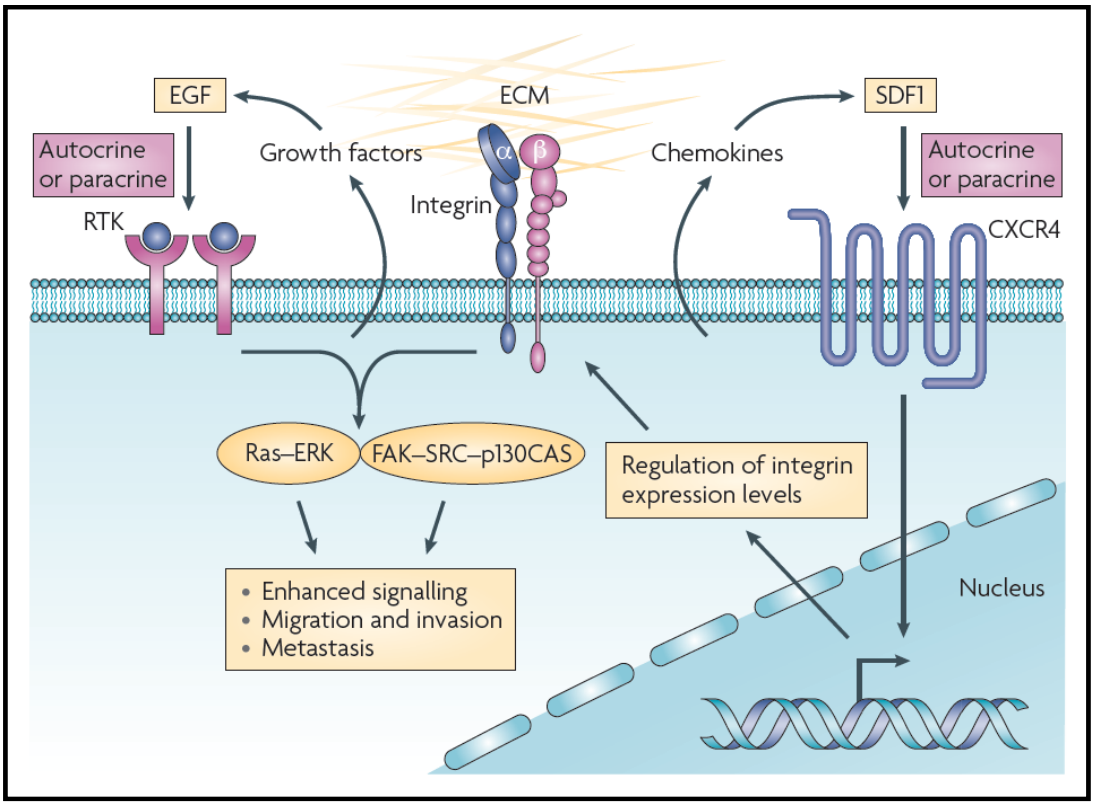

Figure 6. *Integrin-growth factor and integrin-cytokine receptor crosstalk [19].*

The EGF and its receptors (EGFR and ERBB2) are directly involved in tumor formation and metastasis in several tumor types (e.g. breast and pancreatic cancer). In this regard, the cooperation between integrins and EGF receptors affects many aspects of tumor progression, such as initiation, proliferation, migration and invasion.

ERBB2 may cooperate with integrin α6β4 for spontaneous tumor formation and invasion during the onset of breast cancer. The molecular complex formed by integrin α6β4 and ERBB2 promotes the activation of STAT3 and Jun, thus impairing cell polarity and leading to a hyperproliferative phenotype. In addition, the deletion of *Itgb4* might enhance the ERBB2-specific therapy, thus raising the hypothesis that a combination therapy using antagonists targeting integrin α6β4 and EGF could be successful.

The EGF pathway is always up-regulated in pancreatic cancer. As previously underlined, the migration of pancreatic cancer cells on vitronectin is strongly achieved through the cooperation between EGF and integrin αvβ5, which ultimately activates Src signalling. Moreover, EGF promotes colon cancer cells and hepatocellular carcinoma cells migration (through the cooperation with integrins α3β1 and α6β4 or α1β1 and α2β1, respectively).

Integrin ligation alone is able to regulate EGF signalling, given that it can induce EGFR phosphorylation and increase MAPK activation. This is particularly important in breast cancer, because integrin signalling promotes an increase in EGF secretion and the ERBB2 clustering, thus leading to trastuzumab-resistance (trastuzumab is a humanized monoclonal antibody directed toward ERBB2 and preventing uncontrolled cell proliferation).

In summary, the cross-talk between EGF and integrins strongly promotes tumor cells migration, survival and metastatic potential.

Integrin may also cooperate with the HGF receptor MET, promoting tumor progression. The binding between HGF and MET causes the phosphorylation of integrin β4, thus increasing the anchorage-independent growth by means of Src and ERK activation. In this regard, the direct interaction between integrin β4 and MET is able to drive the transformation of fibroblasts. Complex formation between MET and integrin α6β4 strongly enhances HGF-induced signals, potentiating Ras and PI3K signalling. MET may also cooperate with integrin αvβ5 for the expression of genes required for cell migration and directly induced by HGF.

TGF-β is a known inducer of EMT, it is able to enhance cell migration and invasion and is generally secreted as an inactive complex with a LAP. The LAP contains a RGD motif which is bound by several αv integrins. However, only integrins αvβ6 and αvβ8 may activate and regulate TGF-β1. In basal cell carcinoma integrin αvβ6 increases TGF-β1 activation, thus regulating the aggressiveness of the disease. The up-regulation of integrin αvβ6 ultimately leads to EMT and increases cell migration.
Integrins αvβ3 and Src also cooperate with TGF-β to induce EMT of mammary epithelial cells by means of TGFβR2 phosphorylation. In addition, TGF-β stimulation promotes the phosphorylation of integrin β1, thus mediating integrin activation and tumor invasion in a feedback loop.

The cross-talk between integrins and growth factors plays an important role also in the biology of several host cell types, with a particular focus on endothelial cells migration, proliferation and survival. This cooperation is able to protect endothelial cells from distinct apoptotic stimuli, both intrinsic (hypoxia and nutrient deprivation) and extrinsic (by inflammatory mediators such as TNF-α). Raf signalling pathway is the common downstream target of FGFR-integrin αvβ3 and VEGFR2-integrin αvβ5 cooperation. The FGFR-integrin αvβ3 complex promotes the phosphorylation of specific residues of Raf, whereas the VEGFR2-integrin αvβ5 complex takes advantage of the Src-dependent phosphorylation of Raf. In addition, Src recruitment to VEGFR2 promotes the cross-talk of the receptor with integrin αvβ3 and the Src-dependent phosphorylation of the cytoplasmic tail of integrin β3, thus giving rise to a compensatory mechanism. VEGF regulates by itself the affinity state and the activation of integrin αvβ3, which in turn increases the secretion of VEGF in a feedback loop.
Finally, integrin β4 contributes to FGF mediated angiogenesis. In fact, deletion of the signaling portion of the cytoplasmic tail of integrin β4 strongly decreases FGF-induced angiogenesis and tumor size.

CXCR4 is the receptor for SDF-1 and it is expressed on tumor cells and several tumor-associated cell types. The interaction between CXCR4 and SDF-1 generally promotes tumor cell migration and metastasis. In addition, SDF-1 may up-regulate the expression of integrins α5β1 and αvβ3, thus leading to an increase in cell adhesion and invasion (*in vitro*) and to the onset of experimental metastases (*in vivo*). Further studies are needed, but there are evidences that the increase in integrin ligation and activation may reciprocally regulate CXCR4 levels.

2.1.4 Potential applications of integrins targeting

2.1.4.1 Cancer therapy

Integrins on tumor cells are able to enhance metastasis and to facilitate invasion and movements across blood vessels. Moreover, integrins on endothelial cells may modulate cell migration and survival during angiogenesis. As previously seen, integrins are widely expressed in various cell types. Together with their ability to cross-talk with growth factors, cytokines and their receptors, this evidence points out that integrins are appealing therapeutic targets. Integrin targeting agents might be able to inhibit both tumor cells and tumor-associated host cells, and several integrin targeting methods have been extensively studied in order to treat integrin-related disease. Indeed, few integrin antagonists are currently under evaluation in clinical trials for cancer therapy (**Table III**).

Drug name	Target	Drug type	Trial phase	Tumour type	Manufacturer	Refs
MEDI-522 (Vitaxin, Abegrin)	αvβ3	Humanized antibody	II II II	Metastatic melanoma Metastatic prostate cancer Colorectal carcinoma	Medimmune	93–96
M200 (volociximab)	α5β1	Chimeric mouse–human antibody	II II II	Metastatic melanoma Renal cell carcinoma Non-small-cell lung cancer	Protein Design Labs	106,107
CNTO 95	αvβ3 and αvβ5	Human antibody	I	Advanced refractory cancers	Centocor	99
EMD 121974 (cilengitide)	αvβ3 and αvβ5	Peptide	II II II II II	Metastatic melanoma Metastatic prostate cancer Pancreatic cancer Non-small-cell lung cancer Glioblastoma multiforme	Merck KGaA	101–105
ATN-161	α5β1	Peptide	I/II	Malignant glioma	Attenuon, LLC	109

Table III. *Integrin antagonists tested in clinical trials for cancer therapy [4]. It has to be underlined that EMD121974 (Cilengitide®) is currently tested in a phase III clinical trial for glioblastoma multiforme (CENTRIC trial).*

On the whole, integrin antagonists are agents with a low toxicity *in vivo*, because their targets are molecules activated only in clinical settings or in remodeling tissues. Integrin antagonists comprise function blocking antibodies, synthetic peptides and peptidomimetics. A synopsis of integrin antagonists generated so far for cancer therapy is outlined in **Table IV**.

Synopsis of integrin antagonists

	Commercial name	Scientific name	Targeted integrin	Comments	References
Antibodies	Vitaxin	LM609, MEDI-552	$\alpha_V\beta_3$	Passed phase I, currently in phase II	Brooks et al. 1994a, 1994b, 1995; Gutheil et al. 2000; McNeel et al. 2005; Posey et al. 2001; Wu et al. 1998
	CNTO 95		α_V Family, strong affinity for $\alpha_V\beta_3$ and $\alpha_V\beta_5$	Currently in phase I	Centocor, www.centocor.com; Martin et al. 2005; Trikha et al. 2004
		17E6	α_V Family, strong affinity for $\alpha_V\beta_3$, $\alpha_V\beta_5$ and $\alpha_V\beta_1$		Mitjans et al. 2000, 1995
	ReoPro	7E3(abciximab)	$\alpha_{IIb}\beta_3$ Primarily but also $\alpha_V\beta_3$ and $\alpha_M\beta_2$ (Mac-1)	Passed phase I and II Currently in phase III for the prevention of restenosis	Cohen et al. 2000; Varner et al. 1999
		Ha 31/8	$\alpha_1\beta_1$		Senger et al. 1997, 2002
		Ha 1/29	$\alpha_2\beta_1$		Senger et al. 1997, 2002
		NKI-SAM-1, JBS5, or IIA1	$\alpha_5\beta_1$		Francis et al. 2002; Kim et al. 2000a
	Volociximab	M200	$\alpha_5\beta_1$	Passed phase I, currently in phase II	Protein Design Labs, Inc.; www.pdl.com
Peptides, endogenous		PEX(MMP-2 proteolytic fragment)	$\alpha_V\beta_3$		Brooks et al. 1998, 1996; Pfeifer et al. 2000
		Endostatin (collagen XVIII proteolytic fragment)	$\alpha_5\beta_1$	Currently in phase I	Karihaloo et al. 2001; Karumanchi et al. 2001; Kreuger et al. 2002; O'Reilly et al. 1997; Olsson et al. 2004; Sasaki et al. 1999
		Tumstatin (collagen IV proteolytic fragment)	$\alpha_V\beta_3$		Maeshima et al. 2000; Maeshima et al. 2002
Peptides, synthetic	Cilengitide	EMD121974	$\alpha_V\beta_3$	Passed phase I, currently in phase II	Aumailley et al. 1991; Dechantsreiter et al. 1999; Eskens et al. 2003; Haubner et al. 1997; Haubner et al. 1996; Raguse et al. 2004; Smith 2003
		TP508(thrombospondin short peptide)	$\alpha_V\beta_3$		Tsopanoglou et al. 2004, 2002
		S247	$\alpha_V\beta_3$		Reinmuth et al. 2003
		ATN-161	$\alpha_5\beta_1$		Livant et al. 2000; Stoeltzing et al. 2003
		CRRETAWAC (non-RGD fibronectin short peptide)	$\alpha_5\beta_1$		Kim et al. 2000b; Koivunen et al. 1994; Mould et al. 1998
Peptidomimetics		SCH221153	$\alpha_V\beta_3$ and $\alpha_V\beta_5$		Kumar et al. 2001
		BCH-14661	$\alpha_V\beta_3$ and $\alpha_V\beta_5$		Meerovitch et al. 2003
		BCH-15046	$\alpha_V\beta_3$, $\alpha_V\beta_5$ and $\alpha_5\beta_1$		Meerovitch et al. 2003
		Thiolutin	$\alpha_V\beta_3$ (Indirect), decreases paxillin intracellular levels		Minamiguchi et al., 2001
		SJ749	$\alpha_5\beta_1$		Kim et al. 2000a; Marinelli et al. 2005

Table IV. *Synopsis of integrin antagonists (Adapted from [1]).*

Function blocking antibodies generally display high affinity and specificity. Several monoclonal antibodies have been generated to block integrin-mediated pathways, and some are currently in clinical trials.

c7E3 (abciximab, ReoPro®, Centocor, Inc., Philadelphia, PA, USA & E. Lilly, Indiana) is a mouse/human chimera formed by a conjugation of the variable region of mouse anti-integrin αIIbβ3 monoclonal antibody 7E3 with the constant domain of human IgG. c7E3 blocks integrin αIIbβ3 function and mediates the inhibition of platelet aggregation. This antibody is able to bind also integrin αvβ3 at equivalent affinity, thus impairing integrin αvβ3-mediated vascular endothelial cells and melanoma cells adhesion, migration and survival *in vitro*. c7E3 blocks the bFGF-stimulated proliferation of vascular endothelial cells and impairs tumor angiogenesis and growth in a xenograft model. In addition, the administration of c7E3 in nude rats has been demonstrated to inhibit melanoma formation and growth. c7E3 has been also tested in a large-scale clinical trial on patients surgically treated for coronary artery disease, in order to reduce clinical restenosis.

LM609 is a mouse anti-human monoclonal antibody directed toward integrin αvβ3. The intravenous injection of LM609 is able to reduce tumor angiogenesis and growth, with low effects on pre-existing resting vessels. It has been demonstrated that the humanized version of LM609 (namely etaracizumab, or MEDI-522) has a higher affinity for integrin αvβ3. Etaracizumab is able to impair tumor angiogenesis and growth, but also bone resorption. This seems to suggest that the humanized LM609 could play a pivotal role in the reduction of bone metastases. However, clinical trials highlighted the limitations of etaracizumab for metastatic cancer treatment, given that it is driven against a single integrin target.

Generally malignant metastatic cancers involve a combination of different integrin receptor pathways rather than one integrin-dependent signaling cascade alone. To this reason, CNTO95 antibody was developed. This antibody displays a high affinity for integrins αvβ3 and αvβ5, thus promoting both anti-angiogenic and anti-tumor effects. CNTO95 has been studied in clinical trials, which underlined its very low toxicity. On the whole, CNTO95 is well localized to tumor and its anti-tumor activity may be associated to a reduction of the anti-apoptotic protein bcl-2 expression. In addition, CNTO95 synergistically cooperates and enhances the effects of fractionated radiotherapy.

Volociximab is a monoclonal antibody raised against integrin α5β1, which is generally targeted in order to reduce tumor burden in preclinical models. Volociximab impairs angiogenesis and tumor growth. A phase I clinical trial for advanced solid cancers showed that this antibody had no toxicity and was clinically efficacious. Phase II clinical trials are ongoing.

Finally, a couple of additional monoclonal antibodies showed efficacy in preclinical studies. 17E6 antibody is directed against integrins αvβ3, αvβ5 and αvβ1 and promotes a dramatic inhibition of many malignant cancers. 6.3G9 is a monoclonal antibody raised against integrin αvβ6 and has been demonstrated to be effective in inhibiting the growth of human pharyngeal cancer cells both *in vitro* and *in vivo*. 6.3G9 seems to impair also TGFβ signalling, thus suggesting that its efficacy may require the cross-talk of integrin αvβ6 with TGFβ.

Synthetic peptides mimic the structure of natural integrin binding ligands. The largest class of synthetic peptides so far developed comprises RGD mimic peptides, given that the RGD sequence is commonly found in several ECM glycoproteins (e.g. fibronectin, vitronectin, fibrinogen, von Willebrand factor, osteonectin, thrombospondin, proteolysed collagen and laminin). The binding selectivity of synthetic RGD peptides depends on the conformation of the triple-peptide and on its flanking residues as well. It has been established that linear sequences have low bioavailability, due to their short half-life. Indeed, conformationally constrained sequences and their chemical modifications are able to enhance the binding affinity and bioavailability of the peptides, thus obtaining a selectivity from 10- to 100 fold-higher. Cyclic peptides are generally formed by peptide or disulfide bonds, and competitively block the ligand-receptor interaction.

EMD66203 [cyclo(-Arg-Gly-Asp-D-Phe-Val-)] (Merck KGaA) [3] is one of the first cyclic pentapeptide synthesized. It displayed a nanomolar inhibition of vitronectin binding to integrin αvβ3, with a 100 fold selectivity for integrin αvβ3 compared to that for integrin αIIbβ3. A single injection of EMD66203 was able to mediate the disruption of tumor-induced blood vessels after 24 hours, without any effect on pre-existing

vessels. For this reason, EMD66203 was chemically improved changing the aminoacids flanking the RGD sequence, giving rise to EMD121974.

EMD121794, or Cilengitide® [cyclo(-Arg-Gly-Asp-f-(NMe)Val-)] (Merck KGaA) [25] (**Figure 7A**) was able to inhibit the binding between integrin αvβ3 and vitronectin in a low nanomolar range, without interacting with integrin αIIbβ3. Cilengitide® was active on brain tumors in mice and is currently being tested in phase II clinical trials for lung and prostate cancer. In addition, Cilengitide® demonstrated a promising activity in phase II and III clinical trials performed on patients with late stage glioblastoma, given that it extended survival with low adverse effects. On the whole, Cilengitide® displayed both anti-angiogenic and anti-tumor activity, and it is well known that usually the combination therapy between an anti-angiogenic and a chemotherapeutic drug could be more effective than administering the anti-angiogenic compound alone. Moreover, glioblastomas are aggressive and highly vascularized tumors expressing very high amounts of integrin αvβ3 on both angiogenic blood vessels and tumor cells. The microenvironment of high grade glioblastomas is enriched in vitronectin, and it has been proven that the interaction between integrin αvβ3 and vitronectin strongly increases tumor cell survival and invasion. This is the reason why therapies against glioblastoma achieve a marginal increase in patient survival, which is generally short after the diagnosis. Phase I studies with Cilengitide® in patients with recurrent glioblastoma showed that it was well tolerated and able to give durable responses, by means of reducing brain blood flow and promoting stable disease. Another phase I study was performed treating children affected by refractory brain tumors with Cilengitide®, which displayed anti-tumor efficacy and minimal toxicity. A different phase I/II trial examined Cilengitide® on newly diagnosed glioblastoma patients and showed a 69% progression free survival. It is interesting to underline that all these patients had a lowered expression of MGMT, which is a prognostic marker generally used to select the temozolomide (an oral alkylating agent) responsive patients. This seemed to suggest that the combination therapy Cilengitide®-temozolomide might be efficacious. In fact, the CENTRIC phase III clinical trial is ongoing, which will evaluate the effect of Cilengitide® on the survival of patients with the *MGMT* promoter methylation in combination with temozolomide and radiotherapy. In addition, the CORE phase II clinical trial will establish the effects of Cilengitide® alone in a large cohort of patients carrying unmethylated *MGMT* promoters.

ATN-161 (**Figure 7B**) is a non-RGD-based pentapeptide binding to integrins αvβ3 and α5β1. ATN-161 was able to impair tumor growth, angiogenesis and metastasis in animal models. The co-administration of ATN-161 with fluorouracil has been proven to be effective in reducing mouse colon cancer tumor burden and liver metastasis. It is important to remind that integrin β1 signaling might facilitate resistance to radiation therapy, so a cooperation between ATN-161 and radiation therapy as a treatment is currently being taken into account. In addition, ANT-161 was able to block breast cancer growth and metastasis *in vivo* and phase I clinical trials on advanced solid tumors showed that it might induce prolonged stable disease.

TP508 is a sequence deriving from thrombin and containing RGD, and was developed after the observation that thrombin may bind integrin αvβ3 and that some natural thrombin proteolytic fragments display anti-angiogenic functions. If administered in a soluble manner, TP508 is able to block integrin αvβ3-dependent adhesion.

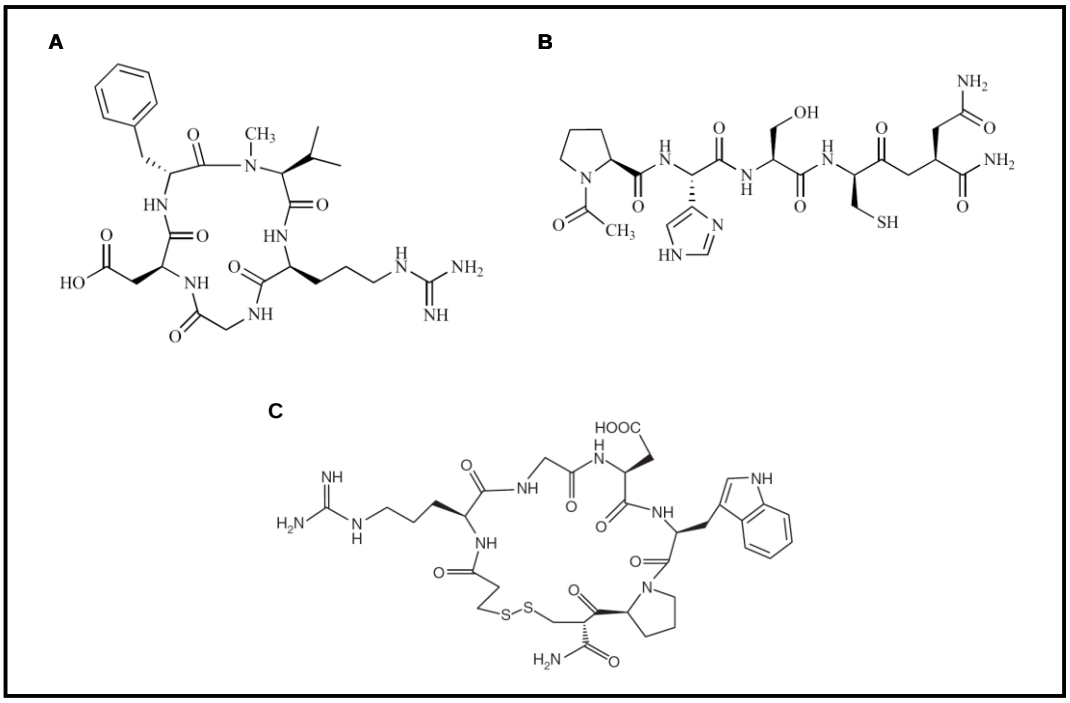

Figure 7. *Examples of integrin antagonists.* ***A:*** *Cilengitide® **B:** ATN-161 **C:** Integrilin® (Adapted from [42] and [51]).*

Other minor integrin antagonist peptide drugs comprise Integrilin®, the cLABL peptide and disintegrins.
Integrilin® (**Figure 7C**) is an antithrombotic drug able to target integrin αIIbβ3 thus blocking platelet aggregation and formation of the complex fibrinogen-integrin αIIbβ3.
cLABL is a peptide derived from integrin LFA-1. LFA-1 binds ICAM-1 and mediates mixed lymphocytes reactions and the hererotypic T-cell adhesion to intestinal mucosa epithelia cell monolayers. Thus, cLABL may potentially target ligands in ICAM-1 highly over-expressing cells.
Finally, disintegrins are low-molecular-mass RGD proteins identified from viper snake and some of them (e.g. trigramin) may inhibit fibrinogen interaction with integrin αIIbβ3.

Peptidomimetics are chemically synthesized products mimicking the functions and structures of the biological integrin antagonists. Peptidomimetics may be obtained through the substitution of one or more peptides by thioamide groups, ketomethylene groups, ore reduced peptide bonds. These modifications determine structural requirements for biological activity, so that the peptidomimetic compounds display higher bioavailability and are able to avoid enzymatic degradation (due to their non-peptidic nature) [25, 51].
As underlined before, RGD is a sequence commonly recognized by integrins on their extracellular ligands. To this reason, a lot of peptidomimetics carrying the RGD sequence in their structure have been synthesized. In particular, after the synthesis of Cilengitide®, the progenitor of such synthetic compounds, studies on peptide secondary structure led to the synthesis of several 6,5- and 7,5-fused 2-oxo-1-azabicyclo[X.3.Ø]alkane amino acids with a conformationally constrained structure. Such compounds were characterized by means of computational and spectroscopic studies, which demonstrated their ability to

mimic those synthesized and studied by Haubner and colleagues. New peptidomimetic compounds display the required activity and selectivity for integrin antagonism [42].
Cyclic-RGD peptidomimetics are particularly relevant to this work. To this reason, a more specific description of such molecules will be outlined in section 2.2.

2.1.4.2 Cancer drug delivery and imaging

Other interesting applications for integrin antagonists are the selective delivery of cytostatic drugs and their diagnostic uses in combination with imaging techniques. It is also possible to conjugate integrin antagonists with therapeutic biomacromolecules, such as endostastin, TNF-α, IL-12, IL-14 or tTF.

Integrins are transmembrane glycoproteins which can be internalized by cells upon activation with anchoring ligands and are over-expressed by angiogenic endothelial cells. These features may be used to facilitate drug delivery in tumor and tumor-associated cells, thus rendering integrins good targets for both anti-tumor and anti-angiogenic therapies [52].
According to the 'vessel normalization hypotesis' promoted by R. Jain (**Figure 8**) [30], the combination of vascular targeting with conventional drugs may facilitate the penetration of the chemicals into tumor, reducing the known adverse effects of classical cytotoxic drugs. In fact, conventional chemotherapeutic drugs are effective but highly toxic, and this evidence limits their clinical efficacy. The directional delivery of such drugs may however circumvent their side effects.

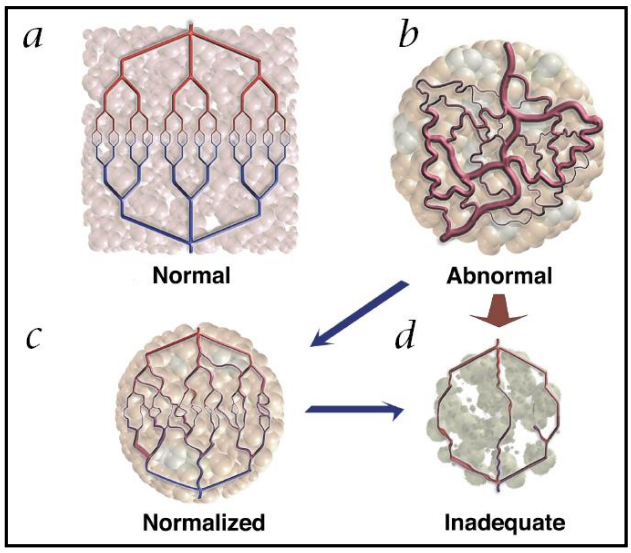

Figure 8. *Schematic of changes in tumor vasculature during the course of anti-angiogenic therapy. (a) Normal vasculature and balance between pro- and anti-angiogenic molecules; (b) abnormal tumor vasculature (increased permeability, vessel diameter, vessel length, vessel density, tortuosity and fluid pressure); (c) direct or indirect anti-angiogenic therapies might prune immature vessels; (d) rapid pruning of tumor vasculature might reduce it, so that vessels are inadequate to support tumor growth. This could promote tumor dormancy [30].*

A couple of tumor vascular homing peptides directed toward the integrin αv were coupled with doxorubicin (**Figure 9**) and were proven to be more effective in the inhibition of tumor growth and lung metastases than doxorubicin alone, with a concomitant less liver and heart toxicity. Moreover, these molecules reduced tumor growth also in integrin αvβ3 low expressing tumors, thus suggesting their addictive anti-angiogenic role.

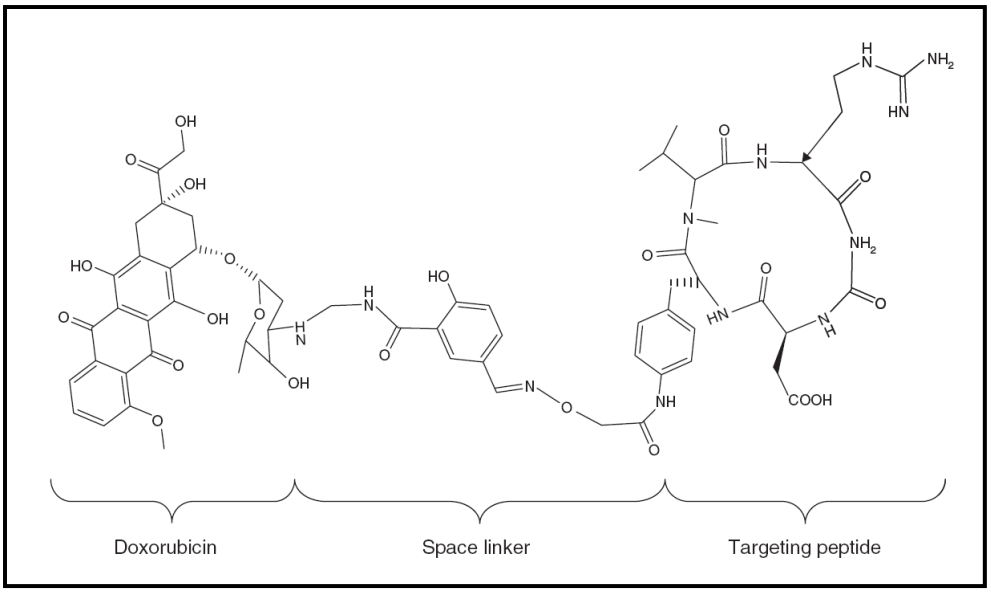

Figure 9. *Doxorubicin-peptide conjugate with a formaldehyde space linker [52].*

Cilengitide® has undoubtedly been demonstrated to be more efficient than the aforementioned peptides, in conjugation with either doxorubicin or taxol. Drug-conjugated Cilengitide® displayed a good inhibition of breast cancer *in vitro*, comparable to that of taxol but with a better uptake kinetic.

Another approach is to use integrin targeting to selectively deliver a gene of interest (e.g. Raf) to angiogenic blood vessels. Hood and colleagues used an integrin αvβ3-coated nanoparticle containing Raf gene and demonstrated that it promoted the apoptosis of tumor associated endothelium and tumor cells, with a sustained regression of the established primary and metastatic tumors.

Finally, integrin-coated nanoparticles represent another promising therapeutic soil, but their biological applications go beyond the aim of this work. However, a particular attention should be pointed to gold nanoparticles, because of their very promising use for medicinal applications. In addition, gold nanoparticles functionalized with the cyclic RGD peptides synthesized by our lab were tested during this work as well (see **Chapter 5**, **paragraph 5.6**).

Traditional imaging techniques are crucial in diagnostics, and gold nanoparticles have been proven to be superior to classic chemical entities. Moreover, nanostructures have been introduced in a broad range of biological applications, such as photodiagnostics and photothermal therapy of cancer and other important diseases.

Gold nanoparticles have considerable applications in optics, catalysis, materials science and nanotechnology, with an obvious resonance on biology and nanomedicine. This is probably due to the fact that oligonucleotides, peptides and synthetic polyethers may be easily attached to gold nanoparticles. Gold nanoparticles present several advantages in biodiagnostics, such as: (i) much reduced or no toxicity; (ii)

much better contrast agents for imaging if compared to organic dyes, which generally suffer from rapid photobleaching; (iii) surface-enhanced and distance- and refractive index-dependent spectroscopic properties.

Interestingly, not only spherical gold nanoparticles have been synthesized, but the shapes of the nanoparticles can be varied according to appropriate techniques as well. Gold nanoparticles conjugation with thiolated polyethylene glycol masks them from the intravascular immune system, thus allowing their multifunctionalization for drug delivery. In addition, gold nanoparticles can be considered a remarkable up-to-date tool as imaging label, contrast agent and cancer diagnosis.

To this regard, it has been highlighted that nanoprobe methods have an application in the detection of single nucleotide polymorphisms and mutations in cancer. Recently, a colorimetric assay was reported for the direct detection of cancer cells by using aptamer-conjugated gold nanoparticles (aptamers are oligonucleic acid or peptide molecules binding to a specific target molecule). It was shown that aptamer-conjugated gold nanoparticles could be assembled on a cell membrane surface for spectral changes, providing a direct visualization of cancer cells. These structures have been applied to leukaemia, lymphoma, lung and liver cancer cells. Moreover, gold nanoparticles covalently conjugated with polyethylene glycol and the monoclonal antibody Herceptin® (enabling the recognition of breast cancer cells expressing specific tumor associated antigens) were shown to be stable and active *in vitro* in the presence of blood and *in vivo* in nude mice model for breast cancer.

Finally, gold nanoparticles have been proven to inhibit angiogenesis, by means of a marked and dose-dependent inhibition of the phosphorylation of proteins responsible for angiogenesis. Gold nanoparticles appear much less toxic if compared with other types of nanoparticles, so that these structures may be reasonably considered an optimal and potential application for the treatment and diagnosis of several diseases [9].

The multimodality imaging performed with small-molecule-based probes is very challenging but rather achievable, because the number of conjugation sites is limited in these structures and a potential interference with receptor-binding affinity may occur. In addition, no validated biomarkers are available to clinically assess the efficacy of anti-angiogenic therapies, so better vascular imaging techniques should be developed in order to monitor the responsiveness to the treatment. To this end, a great effort has been made to characterize integrin antagonists for their ability to specifically deliver diagnostic agents to tumor and tumor-associated host cells (e.g. endothelial cells).

The anti-integrin αvβ3 monoclonal antibody LM609 has been coupled to paramagnetic contrast agents [46] or radionuclides [11] in order to visualize angiogenic vessels (in rabbits and mice). Moreover, these kind of integrin αvβ3-targeted magnetic resonance or imaging vehicles have been proven to be effective in displaying the newly developed vasculature of minute solid tumors in xenograft models [53].

RGD peptides were used as integrin-targeted imaging vehicles as well, due to their overall ability to bind integrin αvβ3. Moreover, several nanoparticle-based RGD-coated probes have been tested, because their large size impairs extravasation and allows a better vascular targeting. In addition, RGD peptides have been labeled with ^{64}Cu [13], ^{18}F [14] or ultrasmall SPIONs [54] for the evaluation of integrin αv expression in mice cancer models (e.g. breast, brain and lung cancer). These molecules are currently under evaluation for their

use in cancer patients. To this regard, scintigraphic imaging by means of a radiolabelled integrin αvβ3-targeted peptide (^{99m}TC-NC100692) was able to image the malignancy in breast cancer patients. Moreover, the delivery of ^{18}F-galacto-RGD during PET allowed a non-invasive quantitative assessment of integrin αvβ3 expression in human tumors [19]. Finally, Bloch and colleagues developed a new molecular construct based on a GRD sequence preferentially driven to integrin β3, in order to image and monitor the functional status of integrin β3 in cells and live animals. This goal was achieved by means of NIR fluorescent probes, thus suggesting to apply this technology to the early manifestations of some tumors (e.g. cervical cancer) [8].

2.2 In-house synthesized RGD integrin ligands

2.2.1 Azabicycloalkane amino acid scaffolds and their biological role

The synthesis of peptidomimetic compounds represents an active and productive field of research in drug design. As underlined above, peptidomimetics display the same biological effects as natural compounds but are metabolically more stable. To this regard, the β3 class of the integrin family gained a special attention in medicinal chemistry, with a particular focus on integrins αIIbβ3 and αvβ3. Because of the established role of integrin αIIbβ3 and αvβ3 in platelet aggregation and vascular or tumor cells biology, respectively, both molecules raised a great pharmacological interest.

As was previously underlined in **sub-paragraph 2.1.4.1**, cyclic RGD peptides have been developed by different groups in order to provide active and selective integrin antagonists. The cyclic template allows a conformational constraint valuable for the bioactive conformation of the molecule which, on the whole, increases the specificity for different integrin receptors [24]. In order to explore the spatial requirements of the antagonist pharmacophore for the selective inhibition of integrins αIIbβ3 and αvβ3, Kessler and co-workers performed a spatial screening based on the synthesis of stereoisomeric cyclic peptide libraries [25]. This procedure led to the synthesis of the highly active integrin αvβ3-selective compound cyclo(-Arg-Gly-Asp-D-Phe-Val-) and of its derivative Cilengitide®, which is currently under evaluation in clinical trials (see **sub-paragraph 2.1.4.1** for further details). Extensive modifications of the lead structure of Cilengitide® were performed in order to reduce the flexibility of the molecule, thus promoting the synthesis of new potent antagonists [18, 34].

In 2000 Belvisi et al. studied peptide secondary structure mimics and reported the synthesis and conformational analysis of a series of 1-aza-2-oxobicyclo[X.3.0]alkane amino acids. The replacement of the D-Phe-Val dipeptide in the lead structure c(RGDfV) with such azabicycloalkane scaffolds showing different reverse-turn mimetic properties may constrain the RGD sequence into different conformations, possibly providing the required activity and selectivity for integrin antagonism and enhancing ligand binding. To this end, a small library of cyclic RGD pseudopentapeptides incorporating stereoisomeric 6,5- and 7,5-fused bicyclic lactams of the general formula provided by **Figure 10A** was synthesized. Some of the RGD cyclic pseudopeptides were examined *in vitro* for their abilities to compete with ^{125}I-echistatin for binding to the purified integrin αvβ3 receptor. The cyclic RGDs were able to efficiently antagonize the highly specific binding between integrin αvβ3 and ^{125}I-echistatin [6].

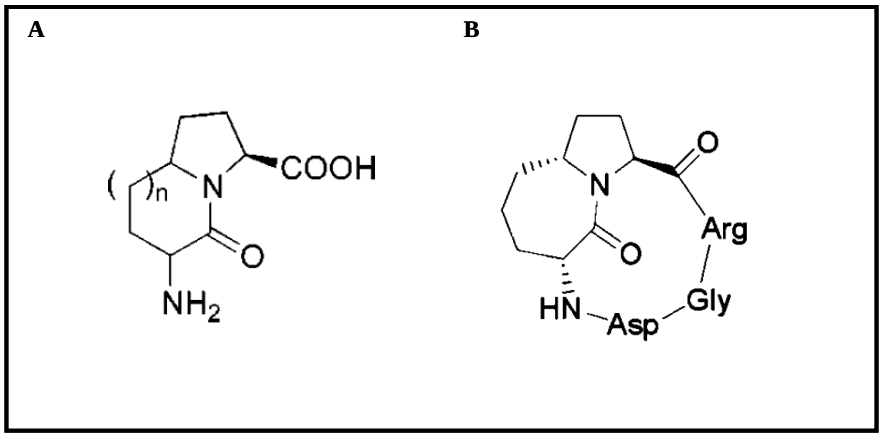

Figure 10. *Cyclic RGD pseudopentapeptides.* ***A:*** *Azabicyclo[X.3.0]alkane amino acids [6];* ***B:*** *Structure of the RGD pseudopeptide ST1646 [7].*

The activity of these cyclic RGD pseudopentapeptides was further evaluated in respect of their putative anti-angiogenic and anti-tumor activity by means of integrin αvβ3 and αvβ5 antagonisms. A particular focus was pointed on the compound ST1646 (**Figure 10B**).

ST1646 was able to inhibit ^{125}I-echistatin binding to both integrins αvβ3 and αvβ5 with low IC_{50} values, and was then tested for its putative activity as an integrin antagonist on *in vitro* and *in vivo* systems too. ST1646 inhibited the adhesion of bovine microvascular cells to immobilized vitronectin or fibronectin in a dose-dependent manner. The compound was tested also on primary vascular endothelial cells (HUVECs, see **Chapter 4**, **paragraph 4.1.2** and **Table V**) and on a panel of human cancer cell lines. ST1646 inhibited cells adhesion to either vitronectin or fibronectin, impairing HUVECs proliferation in a reversible fashion as well.
The *in vivo* activity of ST1646 was evaluated by means of gelatin sponge/CAM assay, using FGF-2 or VEGF as angiogenic stimuli. ST1646 displayed a significant response to the angiogenic boost in both conditions, suggesting its possible dual activity against integrins αvβ3 and αvβ5. In addition, the histological analysis revealed that FGF-2 loaded sponges displayed numerous blood vessels and dense collagenous matrix, in contrast to the absence of blood vessels detectable in FGF-2 plus ST1646-loaded sponges. No cytotoxicity and bleeding-related side effect were observed (**Figure 11 A** and **B**).

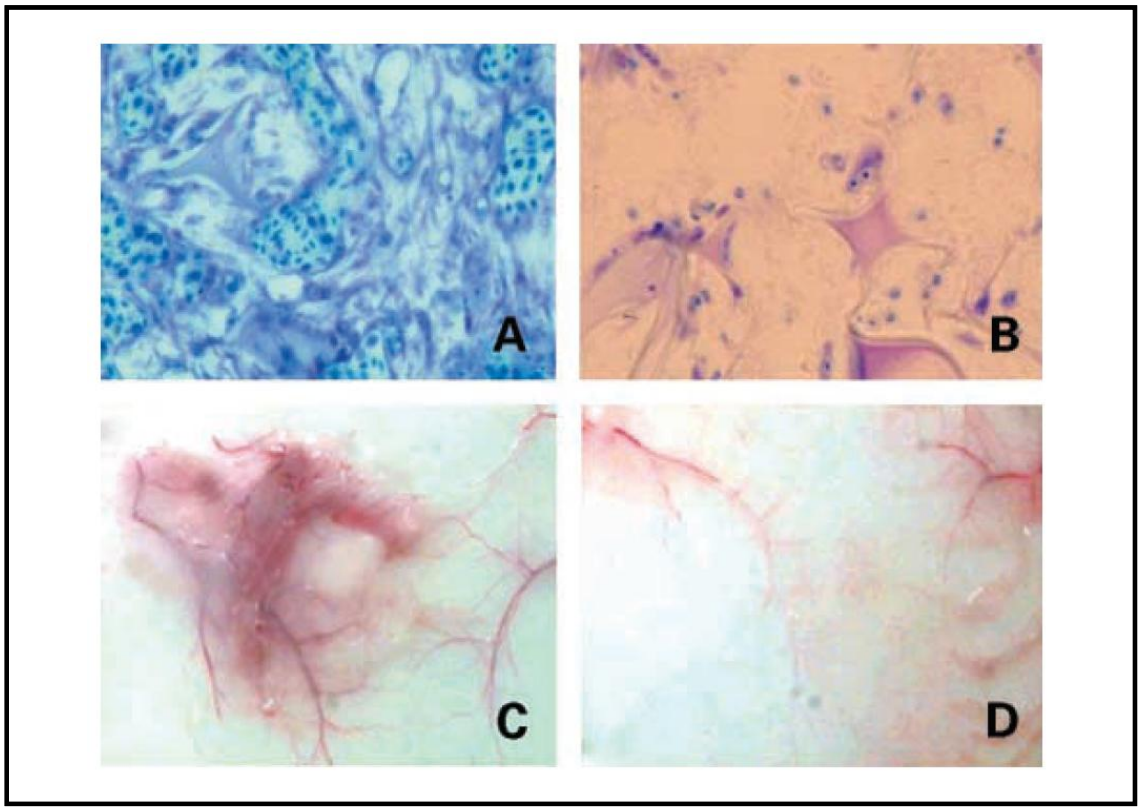

Figure 11. *CAM assay and murine assay with ST1646.* ***A and B:*** *CAM assay. Absence of blood vessels among the sponge* trabeculae *in ST1646-treated implants (B) compared with the significant angiogenic response observed in vehicle-treated samples (A).* ***C and D:*** *Murine dorsal air sac assay. A significant inhibition of tumor growth was observed in ST1646-treated mice (D), in comparison with vehicle-treated animals (C) [7].*

Further, the tumor cell-induced vascularization of the mouse air-sac model allowed to assess the effects of a systemic administration of ST1646 on breast cancer cells-triggered neo-vascularization. The intraperitoneal injection of ST1646 at 15 mg/kg twice daily for 7 days stimulated a 44% reduction of the vascularized area (**Figure 11 C** and **D**).

Finally, the *in vivo* anti-tumor activity of ST1646 was investigated on human ovarian carcinoma cells xenografted in nude mice. Two days after tumor implantation a continuous infusion of ST1646 via an osmotic pump was performed. After 14 days, a significant inhibition of tumor growth and tumor microvessels density was observed. Microvessels density was investigated through the staining of the specific vascular marker CD31 (PECAM-1) (**Figure 12**).

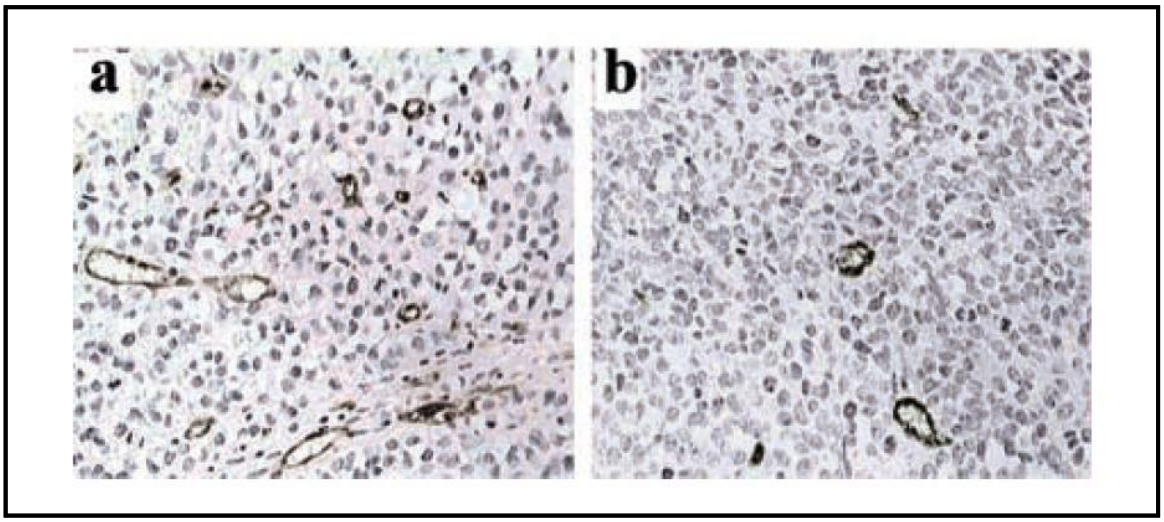

Figure 12. *Anti-tumor and anti-angiogenic activity of ST1646. A significant inhibition of tumor vessels density was observed in ST1646-treated xenografts (b) in comparison with controls (a) [7].*

ST1646 was then demonstrated to provide a cooperative inhibition of tumor cells adhesion, proliferation and angiogenesis, with a high selectivity for integrins αvβ3 and αvβ5 and, to a wider extent, for anchorage-dependent cells [7].

2.2.2 Cyclic RGD-containing functionalized azabicycloalkane peptides

Given that there is a low number of chemical scaffolds suitable for the preparation of conjugated integrin ligands, in 2009 Manzoni et al. reported the functionalization of the previously cited azabicycloalkanes with heteroalkyl side chains ending with a hydroxyl group. This chemical procedure is particularly interesting in that the hydroxyl group can easily be converted into other suitable functional groups (e.g. azide, amine, etc.) or directly used for the conjugation of various chemical entities, for applications in medical diagnosis and therapy. The final aim of this functionalization was to generate high affinity ligands potentially active *per se*, but able to behave as intelligent vectors as well [36].

One particular cyclic-RGD functionalized peptidomimetic, Compound 31, was selected and further studied during this experimental work. This compound contains a conformationally constrained homoSer-Pro dipeptide unit structure (**Figure 13**).

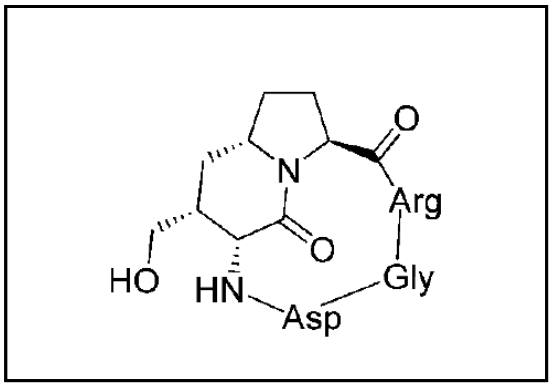

Figure 13. *Compound 31 (Adapted from [36]).*

For a complete description of the synthesis of compound 31 please refer to bibliography [36].

2.2.3 Fluorescein-conjugated cyclic RGD peptidomimetics

Last year our group reported the conjugation of Compound 31 with a fluorescein probe, in order to test its ability as noninvasive imaging agent for the detection of integrin αvβ3 expression. Fluorescein was selected as a probe because it is approved for human use in diagnostic techniques such as retinal angiography [15]. In addition, fluorescein displayed a known safety profile also in humans, and the use of a RGD peptide fluorescein-conjugated in a fluorescent polarization assay has been recently reported [50].

Among all, two compounds were selected and further characterized, namely Compound 13 and 15. Compound 13 and 15 differed upon the nature and the length of the linker conjugating fluorescein with the heteroalkyl side chain of the RGD scaffold. The chemical linker of Compound 15 was longer than that of Compound 13. Moreover, Compound 15 was conjugated with fluorescein through a triazole moiety, while Compound 13 was conjugated through an amide linkage (**Figure 14**).

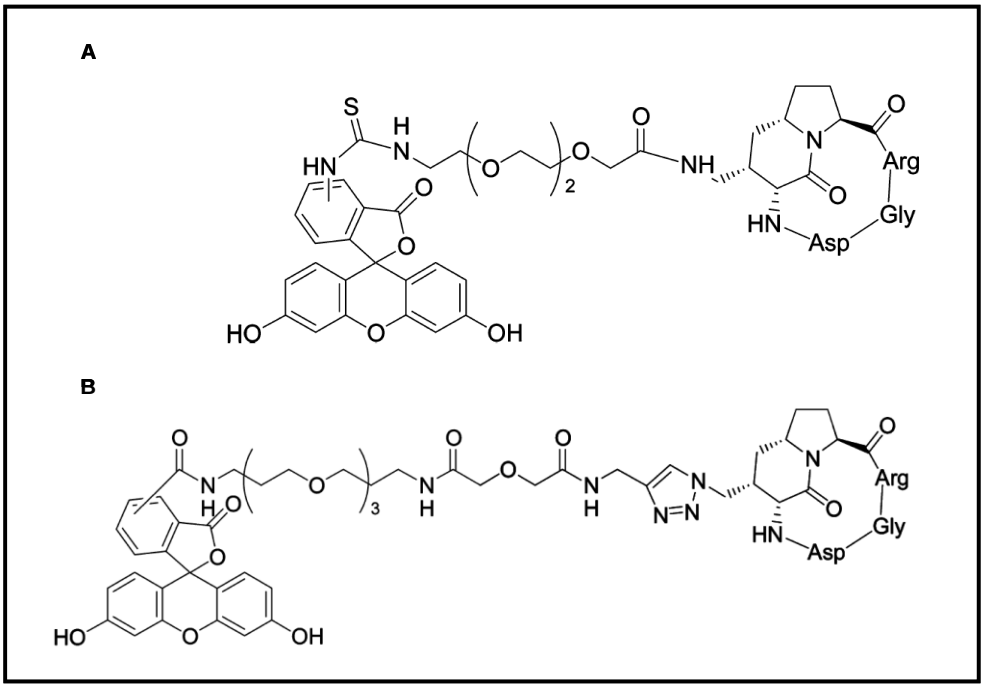

Figure 14. *Chemical structure of Compound 13 and 15. Compound 13 (**A**) and 15 (**B**) differ upon the nature and the length of the linker conjugating the fluorescein moiety with the bicyclic scaffold or RGD ligand (Adapted from [2]).*

Again, for a complete description of the synthesis of the above-mentioned fluorescein-conjugated cyclic RGD peptidomimetics please refer to bibliography [2].

2.2.4 Cyclic-RGD functionalized gold nanoparticles

As previously underlined (see **paragraph 2.1.4.2**), the unique physical and chemical properties of metal nanoparticles recently rendered them a subject of interest in biology and medicine, with a particular focus on applications such as tumor targeting. Among them, gold nanoparticles are particularly interesting for cancer diagnosis and therapy because of their SPR enhanced light scattering and absorption. Moreover, gold

nanoparticles are not susceptible to photobleaching and may convert the strongly absorbed light into localized heat, thus suggesting their possible application in selective laser photothermal therapy. In addition, their tunable size, easy surface chemistry and biocompatibility render gold nanoparticles even more attractive as carriers for drugs or imaging vehicles [9].

Our group recently generated gold nanoparticles functionalized on their surface with a cyclic RGD (cRGD) deriving from Compound 31. The selected integrin ligand was functionalized with a short PEG linker armed with a thiol group. The so-synthesized molecule was employed in the preparation of the nanoparticles. Moreover, in order to visualize the interaction of gold nanoparticles with cells, fluorescein was conjugated as well, giving rise to Compound DA425. During this study, these structures were first tested in solid-phase receptor-binding assay and then on cancer cell lines, in order to determine their putative imaging properties in cancer settings.