Strategies to inhibit tumor associated integrin receptors: rationale for dual and multi-antagonists

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Abstract: The integrins are a family of 24 heterodimeric transmembrane cell surface receptors. Involvement in cell attachment to the extracellular matrix, motility, and proliferation identifies integrins as therapeutic targets in cancer and associated conditions; thrombosis, angiogenesis and osteoporosis. The most reported strategy for drug development is synthesis of an agent that is highly selective for a single integrin receptor. However, the ability of cancer cells to change their integrin repertoire in response to drug treatment renders this approach vulnerable to the development of resistance and paradoxical promotion of tumor growth. Here, we review progress towards development of antagonists targeting two or more members of the RGD-binding integrins, notably $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_5\beta_1$, and $\alpha_{IIb}\beta_3$, as anticancer therapeutics.

Integrin structure and implications for drug development

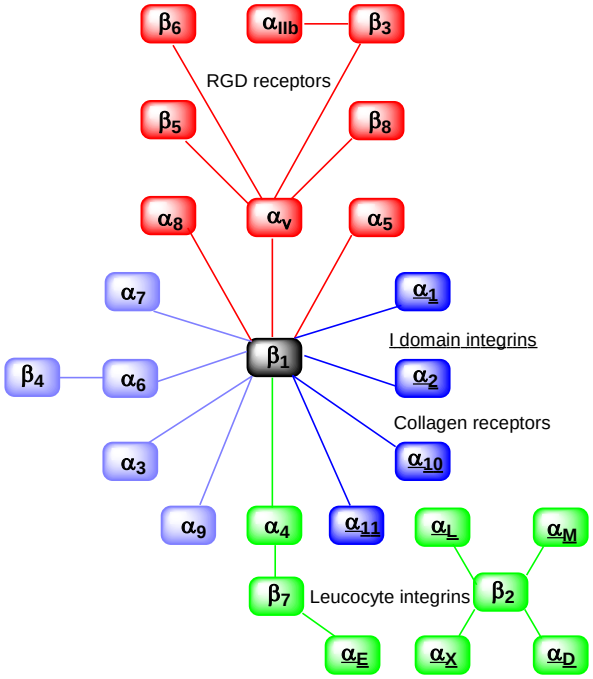


Figure 1 Combinations of integrin subunits which form the 24 human receptors

Recognition sequence	Integrins	Major Ligands
RGD	$\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_{IIb}\beta_3$	Vitronectin, Fibronectin, Osteopontin, Fibrinogen
LDV and related sequences	$\alpha_4\beta_1$, $\alpha_9\beta_1$, $\alpha_4\beta_7$, $\alpha_E\beta_7$, $\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, $\alpha_D\beta_2$	Fibronectin, Vascular Cell Adhesion Molecule 1, Mucosal Addressin Cell Adhesion Molecule-1, Intercellular Cell Adhesion Molecule-1
GFOGER	$\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, $\alpha_{11}\beta_1$	Collagen, Laminin
Other	$\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_6\beta_4$	Laminin

Table 1 Integrin ligands and recognition sequences

The integrin family comprises 24 transmembrane receptors, each a heterodimeric combination of 1 of 18 α and 1 of 8 β protein subunits. Their function is to integrate adhesion and interaction with the extracellular microenvironment with intracellular signalling and cytoskeletal rearrangement through transmitting signals across the cell membrane on ligand binding. They are usually classified into subfamilies based on the major protein recognised, type of cell expressing the receptor, or the presence of an I-domain in the α subunit (Figure 1). The largest subfamily recognise the RGD tripeptide found in a range of extracellular matrix (ECM) ligands (Table 1), although these integrins also contain interaction sites for other proteins such as matrix metalloproteinases, and are able to bind other sequences. Integrins involved in immune functions recognise LDV and related sequences such as LDT and IDS. A subset of the collagen and laminin binding integrins recognise GFOGER, and the remaining laminin binding integrins recognise a range of peptide sequences with no common features currently reported.[1]

The binding site for RGD and related sequences is located at the junction of the α and β subunits of RGD-binding integrins such as $\alpha_v\beta_3$ and $\alpha_5\beta_1$. Key features of the RGD binding site are shown in Figure 2. Ligand binding has been explored through NMR studies, mutagenesis, X-ray structures of bound ligands and homology modelling.[2,3] Ligand binding to the β subunit is primarily through an electrostatic interaction between a carboxylate group on the ligand and a positively charged metal ion (usually Mg^{2+}) associated with the integrin subunit. The α subunit binds the basic arginine sidechain through interactions with several subunit specific acidic residues.

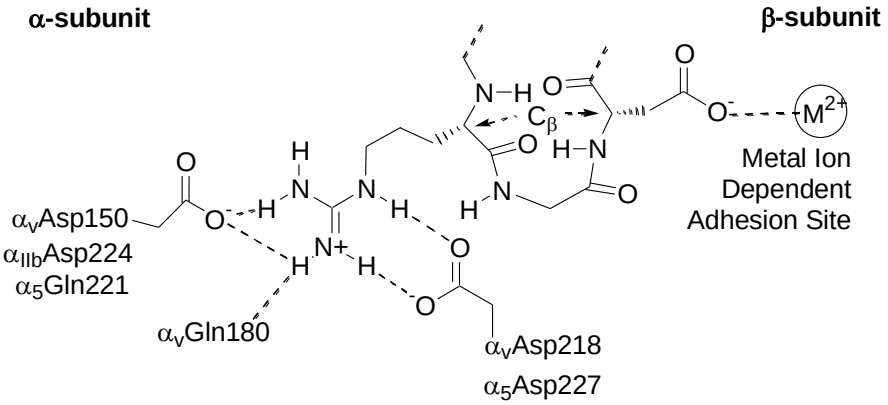


Figure 2 Key features of the RGD binding site

Differences in binding site size and the identity of the arginine-binding residues have allowed development of integrin specific ligand mimetics which act as competitive antagonists; however, the information amassed in determining ligand specificity should also be applicable to the design of multi-integrin antagonists. NMR studies on peptides have shown that molecules binding to $\alpha_{IIb}\beta_3$ typically have a distance of 7.5-8.5 Å between the β carbons of arginine and aspartate, and compounds binding $\alpha_v\beta_3$ and $\alpha_5\beta_1$ less than 6.7 Å.[4] For peptidomimetic ligands, it is easier to measure the distance between acidic and basic groups acting as sidechain mimetics; typically 13.5-15.5 Å is required for anti-$\alpha_{IIb}\beta_3$ activity and 10.0-13.8 Å for anti-$\alpha_v\beta_3$.[5,6] $\alpha_5\beta_1$ also binds molecules spanning ~13 Å, although its binding site is larger than $\alpha_v\beta_3$ with space for bulky residues adjacent to the RGD site and allowing more lipophilic interactions.[7] $\alpha_5\beta_1$ binding to the arginine sidechain has similarities to both α_{IIb} and α_v.[3] $\alpha_v\beta_5$ is highly similar to $\alpha_v\beta_3$ in the ligand binding site region although homology modelling suggests the $\alpha_v\beta_5$ binding pocket is somewhat smaller and cannot accommodate large groups adjacent to the metal-ion dependent adhesion site.[8] The structures of the other RGD-binding integrins, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_v\beta_1$ and $\alpha_8\beta_1$, have not yet been studied in detail, although two recently reported homology models for $\alpha_v\beta_6$[9,10] provide an important starting point to develop an understanding of antagonist selectivity. It has been shown experimentally that the common α_v subunit and distinct β subunits allow the synthesis of small molecules with

varying degrees of selectivity within the subfamily. $\alpha_8\beta_1$ has not yet been proven to be a target for therapeutic intervention. It has some functions in common with other members of the subfamily, suggesting there may be some value in multi-targeting, but further investigations are required to establish whether this will be safe, effective, and possible.

Integrins possess redundancy in ligand recognition, adhesion and signalling functions, and cells invariably express multiple integrins from several subfamilies. Crosstalk between integrins can affect cell functions, for example *via* trans-dominant inhibition where competition for intracellular signalling effectors means inhibition of the function of one integrin promotes activation, adhesion and signalling of another.[11]

Despite the intense interest in integrins as targets in a range of diseases,[12,13] the main strategy to date has been to develop a highly selective antagonist of a single integrin, whilst the deliberate development of dual antagonists has been little explored,[14] with the exception of the application of $\alpha_4\beta_1/\alpha_4\beta_7$ antagonists in autoimmune disorders.[15] The drive towards selectivity for a single integrin may reduce the effectiveness or safety of the developed antagonists, leaving them vulnerable to the development of resistance, or even paradoxical effects, as targeting one receptor promotes the upregulation of a related receptor binding the same ligand to maintain adhesion and signalling.

This Perspective review summarises evidence of the importance, and interactions, of pairs and wider combinations of integrins in cancer progression and dissemination, and progress in the development of dual and multi-antagonists to efficiently target these processes.

Dual antagonism

$\alpha_{IIb}\beta_3/\alpha_v\beta_3$

$\alpha_{IIb}\beta_3$ is normally expressed only on platelets and megakaryocytes. On platelet activation by any agonist, $\alpha_{IIb}\beta_3$ is activated and binds fibrinogen through its RGD and KQAGDV motifs

causing platelet crosslinking thus thrombus formation.[16] Antagonism of $\alpha_{IIb}\beta_3$ prevents platelet adhesion and aggregation, therefore it has been a popular target in the development of broad spectrum anti-thrombotic therapy.[17] Three $\alpha_{IIb}\beta_3$ antagonists are currently approved for clinical use in the treatment of acute coronary syndrome and during percutaneous coronary intervention; the cyclic peptide Eptifibatide, small molecule Tirofiban and antibody Abciximab. Unlike the former two, Abciximab is not selective [IC_{50} (cell adhesion) $\alpha_{IIb}\beta_3$ 6.5 nM, $\alpha_v\beta_3$ 9.8 nM, $\alpha_M\beta_2$ 160 nM][18] and its ability to antagonise both β_3 integrins and potentially reduce inflammation has been suggested as the reason for its better performance at preventing restenosis compared to $\alpha_{IIb}\beta_3$-specific antagonists.[19,20]

The most important sites of expression of $\alpha_v\beta_3$ are osteoclasts, where it controls attachment to the bone surface to form a sealing zone for bone resorption,[21] and on active endothelial cells where it mediates vascular angiogenesis.[22] The role of integrins in angiogenesis is complex; antagonism or knockout of $\alpha_v\beta_3$ can promote as well as inhibit angiogenesis, but a recent study has confirmed that $\alpha_v\beta_3$ is a valid target for preventing the early stages of angiogenesis.[23]

$\alpha_v\beta_3$ is expressed on a wide range of tumors and associated vasculature, where it is associated with invasion, metastasis and poor prognosis.[24-27] $\alpha_v\beta_3$ promotes site-specific metastasis to the lungs and bone; adhesion to fibronectin, vitronectin, osteopontin or bone sialoprotein allows the metastatic deposit to become established[28-32] and tumoral $\alpha_v\beta_3$ signalling is required for bone deposition in osteoblastic lesions, in contrast with the recognised role of osteoclast $\alpha_v\beta_3$ in osteolytic metastases.[30,33-35]

$\alpha_{IIb}\beta_3$ is abnormally expressed in melanoma and prostate tumors and is associated with increased tumor growth, recurrence and metastasis.[36-38] Cells co-expressing $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ show increased growth and angiogenesis *in vivo*, but reduced $\alpha_v\beta_3$ function due to its displacement or trans-dominant inhibition by $\alpha_{IIb}\beta_3$, suggesting that selectively targeted anti-$\alpha_v\beta_3$ antagonists will prove clinically ineffective in dual β_3 expressing tumors.[36,39]

The full potential of dual $\alpha_v\beta_3/\alpha_{IIb}\beta_3$ integrin antagonism is evident when considering their roles in metastasis. β_3 integrins promote lymphatic metastasis, and hematogenous metastasis through promoting extravasation from the primary tumor, cell adhesion, intravasation and tumor growth at the metastatic site. β_3-mediated interactions between tumor cells and platelets promote platelet aggregation and release of growth factors, and increase cell survival through formation of a tumor-platelet microthrombus which protects circulating tumor cells from immune targeting and facilitates arrest and adhesion in blood vessels.[40-45]

Both selective anti-$\alpha_{IIb}\beta_3$ antagonists, and $\alpha_v\beta_3$ antagonists have shown a wide range of anticancer effects (for a previous review see [26]). Studies using Abciximab and related murine antibodies are particularly important in demonstrating the potential of dual β_3 antagonism, although the problems of immunogenicity and bleeding associated with Abciximab suggest further development of small molecules is required to provide an attractive clinical candidate. Dual β_3 antagonism with these antibodies was effective at blocking tumor growth and angiogenesis through targeting tumor cells' interaction with platelets and endothelial cells, in addition to direct effects on tumor tissue.[46-48] In a model of bone metastasis, daily treatment with m7E3 F(ab')2 was effective at reducing growth of β_3 negative tumors implanted in the tibia through acting on platelets and the bone microenvironment, indicating that this target will be applicable to a wide range of tumors.[47] m7E3 F(ab')$_2$ pretreatment was also shown to reduce the development of lung metastases after intravenous injection of tumor cells as a model of the early stages of hematogenous dissemination.[49] Combining two small molecule $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ antagonists has also been shown to be more effective than either agent alone at preventing tumor cell adhesion to endothelial cell or ECM.[50,51]

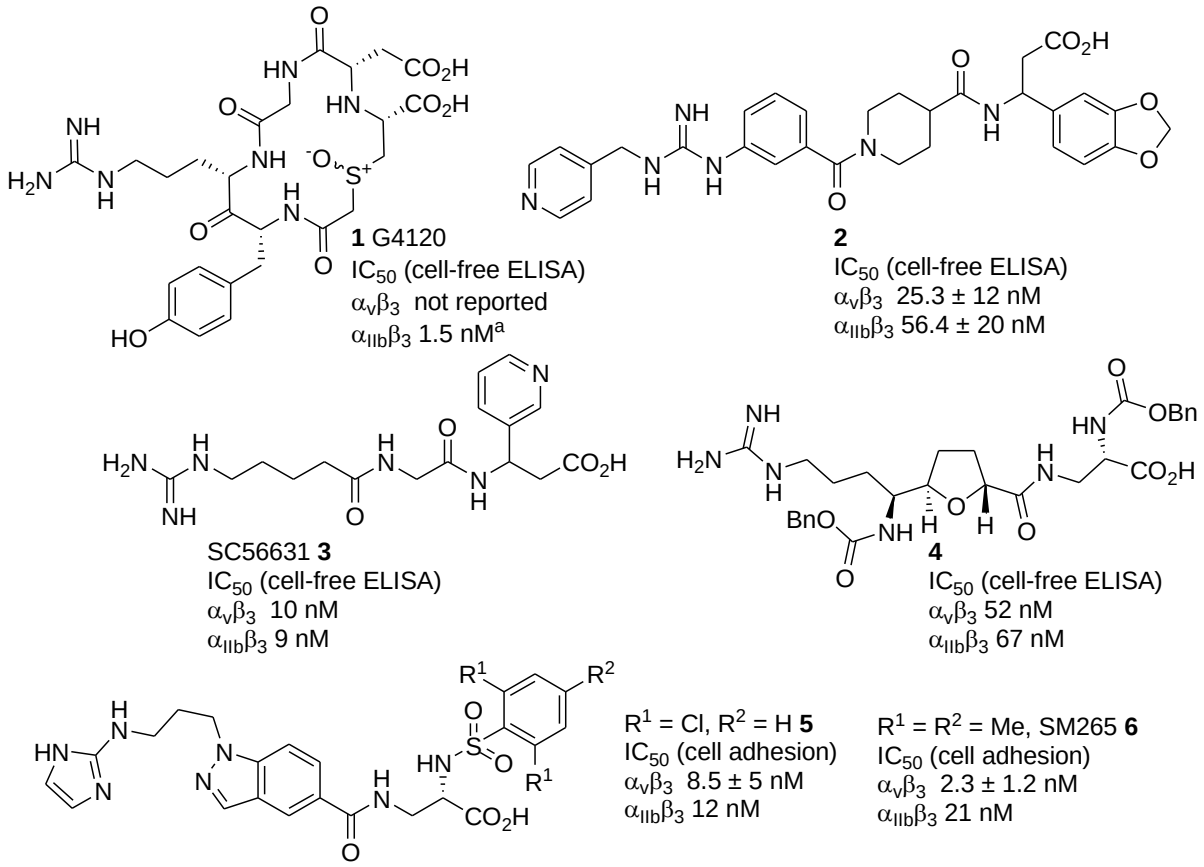
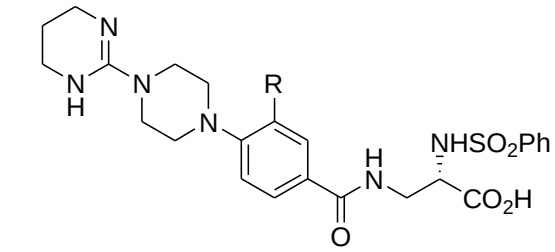


Figure 3 $\alpha_{IIb}\beta_3/\alpha_v\beta_3$ antagonists [a.]The absence of standard deviation from a measurement indicates this data was not reported in the original reference.

Dual antagonists have been obtained incidentally during the development of highly active selective $\alpha_{IIb}\beta_3$ or $\alpha_v\beta_3$ antagonists eg **1**-**4** (Figure 3).[52-54]

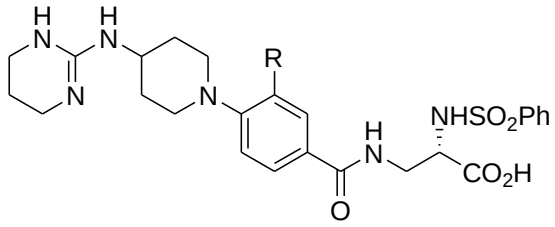
The cyclic peptides G4120 **1** and G3580 (a related structure which unfortunately cannot be unambiguously identified)[55] were found to be non-specific[56] and have been used as tools in integrin biology and to demonstrate that inhibition of both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ was effective at reducing restenosis, although there was no advantage in using G3580 [IC_{50} (cell free ELISA) $\alpha_{IIb}\beta_3$ 1.5 nM; $\alpha_v\beta_3$ 8 nM)] compared to a highly selective $\alpha_{IIb}\beta_3$ or $\alpha_v\beta_3$ antagonist alone.[57] SC56631 **3**, developed in an anti-$\alpha_v\beta_3$ antiosteoporotic programme, has been used as a tool compound to demonstrate that $\alpha_v\beta_3$ antagonism is cytotoxic to cysteine-rich protein 61 overexpressing cancer cells which depend on integrin survival signalling; in this $\alpha_{IIb}\beta_3$ negative *in vitro* model it was less effective when compared to antagonists with subnanomolar IC_{50}s on $\alpha_v\beta_3$.[58] SM265 **6** has demonstrated anticancer effects *in vitro* and *in*

vivo through binding to $\alpha_v\beta_3$ but possible contribution of $\alpha_{IIb}\beta_3$ to its *in vivo* efficacy has not been investigated.[59,60]



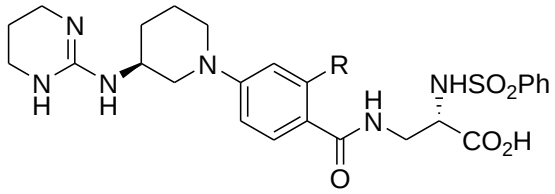
R = H, **7**
IC_{50} (cell free ELISA)
$\alpha_v\beta_3$ 160 nM
$\alpha_{IIb}\beta_3$ 0.73 nM

R = Cl, **8**
IC_{50} (cell free ELISA)
$\alpha_v\beta_3$ 3.6 nM
$\alpha_{IIb}\beta_3$ 0.12 nM
VSMC/Vn 4200 nM



R = H,**9**
IC_{50} (cell free ELISA)
$\alpha_v\beta_3$ 1.3 nM
$\alpha_{IIb}\beta_3$ 3.1 nM
VSMC/Vn 190 nM

R = OMe, **10**
IC_{50} (cell free ELISA)
$\alpha_v\beta_3$ 0.19 nM
$\alpha_{IIb}\beta_3$ 0.44 nM
VSMC/Vn 110 nM



R = H, **11**
IC_{50} (cell free ELISA)
$\alpha_v\beta_3$ 0.48 nM
$\alpha_{IIb}\beta_3$ 0.56 nM
VSMC/Vn 31 nM

R = F, **12**
IC_{50} (cell free ELISA)
$\alpha_v\beta_3$ 0.23 nM
$\alpha_{IIb}\beta_3$ 0.78 nM
VSMC/Vn 16 nM

Figure 4 MN447 and related antagonists

MN447 **10** (Figure 4) is the only small molecule deliberately developed as a dual β_3 antagonist, intended as a small molecule analogue of Abciximab for the treatment of acute thrombotic events. Starting from a selective $\alpha_{IIb}\beta_3$ antagonist **7**, altering substituents on the central aromatic ring gave compound **8** with nanomolar anti-$\alpha_v\beta_3$ and anti- $\alpha_{IIb}\beta_3$ activity in cell free ELISA but relatively ineffective at inhibiting $\alpha_v\beta_3$-mediated cell adhesion to vitronectin.[61] *Para*-substitution about the central aromatic ring is required for $\alpha_{IIb}\beta_3$ activity; meta-substitution shortens the molecule thus yielding selective $\alpha_v\beta_3$ antagonists.[62] Changing to a 4-aminopiperidine linker **9** improved the anti-adhesion activity,[61] and adding either R = F, OH or OMe further increased potency and resulted in good water solubility.[63] 3-aminopiperidine also afforded a highly active scaffold eg **11, 12**.[64] MN447 **9** (R = OMe) was effective in treating thromboembolus-induced dysfunction of the heart muscle *in vivo* and is

currently in preclinical development. Importantly, both MN447 **10** and SC56631 **3** have been demonstrated not to prolong bleeding time.[63,65]

$\alpha_v\beta_3/\alpha_v\beta_5$

$\alpha_v\beta_5$ has a similar expression pattern and function to $\alpha_v\beta_3$; both are highly expressed by activated endothelial cells and have similar roles in angiogenesis, promoting the angiogenic response to different growth factors.[66] $\alpha_v\beta_5$ has been shown to be highly expressed on a wide range of tumor types in both cell lines and clinical material;[67] this ubiquitous expression suggests it may be a good target receptor for developing tumor-directed therapies to benefit the widest range of patients. However, a number of tumors and associated vasculature co-express $\alpha_v\beta_3$ and $\alpha_v\beta_5$;[68-70] given that the two integrins engage the same ECM ligands and activate complementary cell signalling pathways[71-73] to promote tumor progression, dual targeting will be necessary to effectively treat such tumors.

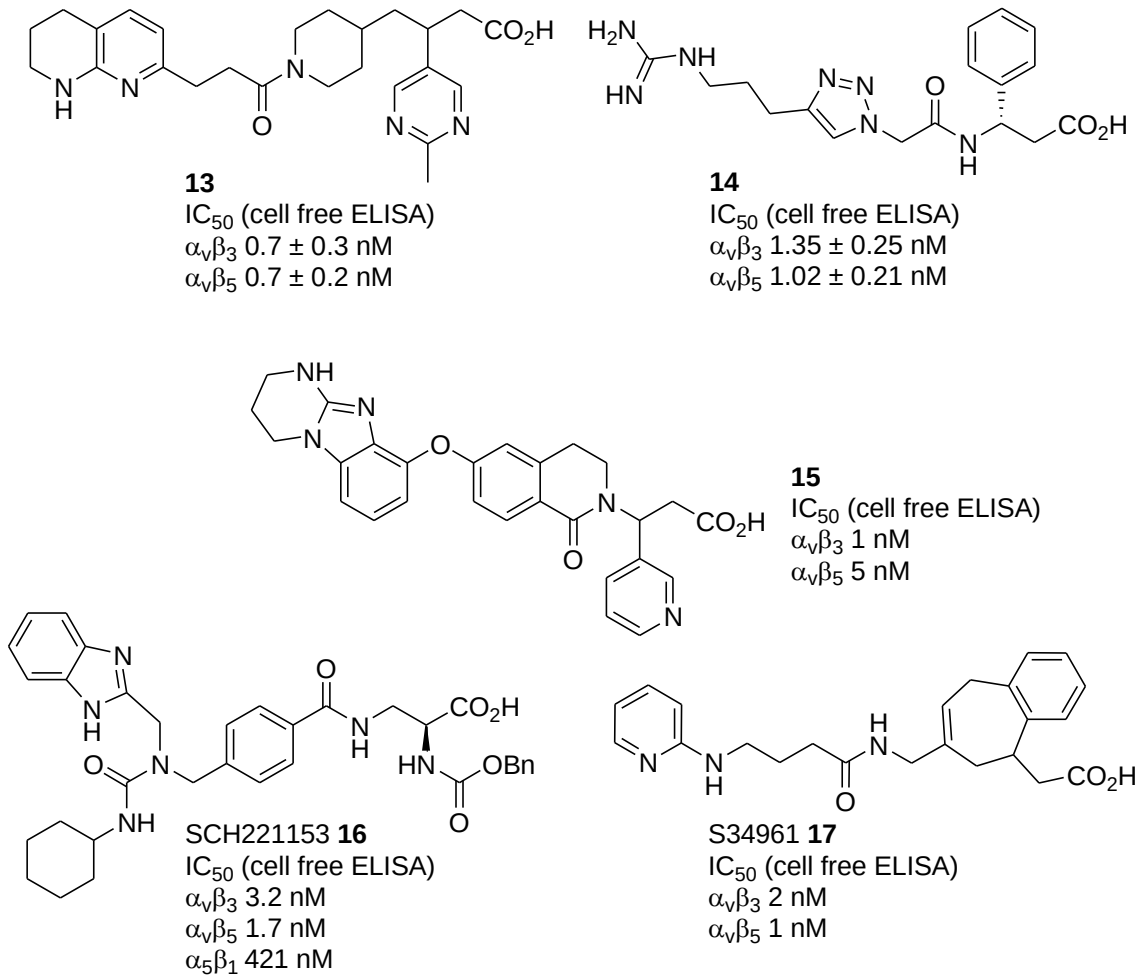


Figure 5 $\alpha_v\beta_3/\alpha_v\beta_5$ antagonists

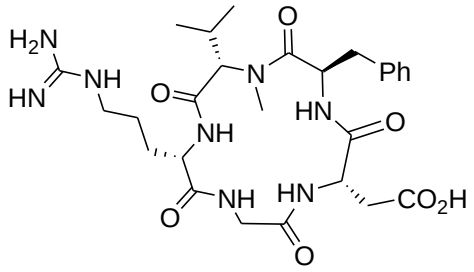
Screening of compounds which are highly active against $\alpha_v\beta_3$ reveals they frequently also possess similar activity against $\alpha_v\beta_5$ eg **13**, **14** (Figure 5).[53] Alternatively, dual antagonists have been deliberately prepared through screening to identify and optimise compounds with similar affinity for both integrins eg **15**.[74] **14** is antiangiogenic and is highly effective at preventing adhesion of $\alpha_v\beta_3/\alpha_v\beta_5$ expressing melanoma cells to a range of RGD-containing ECM ligands, particularly osteopontin, indicating that it may be effective in reducing bone metastasis. It appears to be selective for inhibiting the active form of the integrin. Decreasing the length of the guanidine-bearing sidechain gave an $\alpha_v\beta_5$-selective agent.[75]

SCH221153 **16** was developed by screening a combinatorial RGD-mimetic library. The diaminopropionic acid aspartate mimetic was identified as essential for activity, and compounds optimised for high $\alpha_v\beta_3$ activity, resulting in the selective dual $\alpha_v\beta_3/\alpha v\beta_5$

antagonist **16**.[76] Despite a short half-life (12 minutes), **16** significantly reduced the growth of α_v-negative melanoma xenografts, indicating that continuous exposure to high blood levels of integrin antagonist is not required to effectively inhibit angiogenesis, and contrasting with a subsequent report that drops in blood levels of an $\alpha_v\beta_3$ antagonist to subtherapeutic concentrations promote tumor growth.[77]

S34961 **17** was developed using the cycloheptene ring system to mimic the conformation of the RGD motif in natural integrin ligands. Other arginine sidechain mimetics such as tetrahydropyrimidine, tetrahydronaphthyridine, imidazoline and benzimidazoline also gave high dual antagonist activity.[78] *In vitro*, **17** caused cell detachment and sensitized quiescent colon cancer cells to mitogen-activated protein kinase kinase inhibition by reducing integrin survival signaling.[79] The resolved *S* isomer of **17** (S 36578-2) was shown to cause anoikis of endothelial cells.[80]

Cilengitide



Cilengitide **18**
IC_{50} (cell-free ELISA)
$\alpha_v\beta_3$ 0.65 ± 0.07 nM
$\alpha_v\beta_5$ 11.7 ± 1.5 nM
$\alpha_5\beta_1$ 13.2 ± 0.6 nM

Figure 6 Cilengitide

The cyclic pentapeptide Cilengitide **18** (Figure 6) is the first non-antibody integrin antagonist to progress to Phase III clinical trials for the treatment of cancer. The development of Cilengitide has been previously described by its inventors,[81] so will be briefly summarised here. Briefly, Cilengitide was derived from the cRGDfV pentapeptide by *N*-methylation;

methylation of valine was found to be the preferred position for increasing activity against $\alpha_v\beta_3$ whilst maintaining selectivity over $\alpha_{IIb}\beta_3$.[82,83] Cilengitide is less selective against other RGD-binding integrins; it is usually described as an $\alpha_v\beta_3/\alpha_v\beta_5$ antagonist but has similar activity (12 nM) against both $\alpha_v\beta_5$ and $\alpha_5\beta_1$. Addition of a second *N*-methyl group to the glycine, aspartic acid or D-phenylalanine residue improved the $\alpha_v\beta_3$ selectivity of the peptide.[84]

The progress of Cilengitide through clinical trials was most recently summarised by Scaringi *et al*.[85] It showed a good safety profile in all applications, and reached Phase III clinical trials against glioblastoma. Unfortunately, this first Phase III trial recently failed to reach its primary endpoint of increased progression-free survival, discouraging further development.[86] Preclinical and some clinical results indicate that Cilengitide should be used as a combination with radiotherapy or other chemotherapies.[87] Notably, combination with cytotoxic chemotherapy in head and neck squamous cell carcinoma achieved 100% disease control in the Phase I cohort.[88] Other preclinical studies indicate it may be effective in preventing or treating bone metastasis.[89,90]

$\alpha_v\beta_3/\alpha_5\beta_1$

Tumors often overexpress $\alpha_5\beta_1$ along with $\alpha_v\beta_3$ and other α_v integrins.[91,92] Both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ allow interaction between cancer cells, endothelial cells and the ECM through common ligands, most notably fibronectin.[93] $\alpha_v\beta_3$ has been shown to regulate the function of $\alpha_5\beta_1$ and *vice versa*; selective inhibition of $\alpha_v\beta_3$ allows $\alpha_5\beta_1$ mediated adhesion but not migration, whereas inhibition of both integrins more effectively prevented cancer cell adhesion.[94,95] $\alpha_5\beta_1$ binding fibronectin promoted endothelial cells binding to vitronectin, and angiogenesis.[96]

Inhibition of β_1 integrins has been shown to promote tumor progression and metastasis by increasing the expression of β_3 indicating that selective inhibition of $\alpha_5\beta_1$ is likely to be ineffective as an anticancer therapy due to β_3-mediated resistance. However, $\alpha_v\beta_3$ antagonism

or combination β_1/β_3 antagonism is not vulnerable to this resistance.[97] In contrast, $\alpha_v\beta_3$ antagonism can increase tumor cell invasiveness in the presence of high levels of fibronectin due to increased $\alpha_5\beta_1$ recycling; the effect of dual β_1/β_3 antagonism in these circumstances remains to be established.[98] Such changes in receptor expression and usage in response to the chemical environment of a tumor may explain the contradictory results obtained in a minority of pathological studies, which show decreased expression of $\alpha_5\beta_1$ as disease progresses.

$\alpha_5\beta_1$ has a similar proangiogenic function to $\alpha_v\beta_3$ and knock-out of both α_5 and α_v is required to prevent vascular development since loss of one is generally compensated by the other.[99] Changes in the conformation of fibronectin favor binding by $\alpha_v\beta_3$ rather than $\alpha_5\beta_1$ in the tumor microenvironment and promote vascular endothelial growth factor (VEGF) secretion; selective $\alpha_5\beta_1$ inhibition stimulated VEGF secretion, but $\alpha_v\beta_3$ inhibition reduced it, suggesting that targeting $\alpha_5\beta_1$ alone may have undesirable pro-angiogenic effects but targeting $\alpha_5\beta_1/\alpha_v\beta_3$ should not.[100]

Selective $\alpha_5\beta_1$ and $\alpha_v\beta_3$ antibodies had little effect on angiogenesis in the stromal microenvironment as single agents, but combination of selective antibodies completely blocked endothelial tube formation.[101] Taken together, biological studies suggest that the safety and effectiveness of selective anti-$\alpha_5\beta_1$ therapy requires further evaluation, whereas targeting $\alpha_5\beta_1$ in combination with $\alpha_v\beta_3$ is likely to prove more efficient.

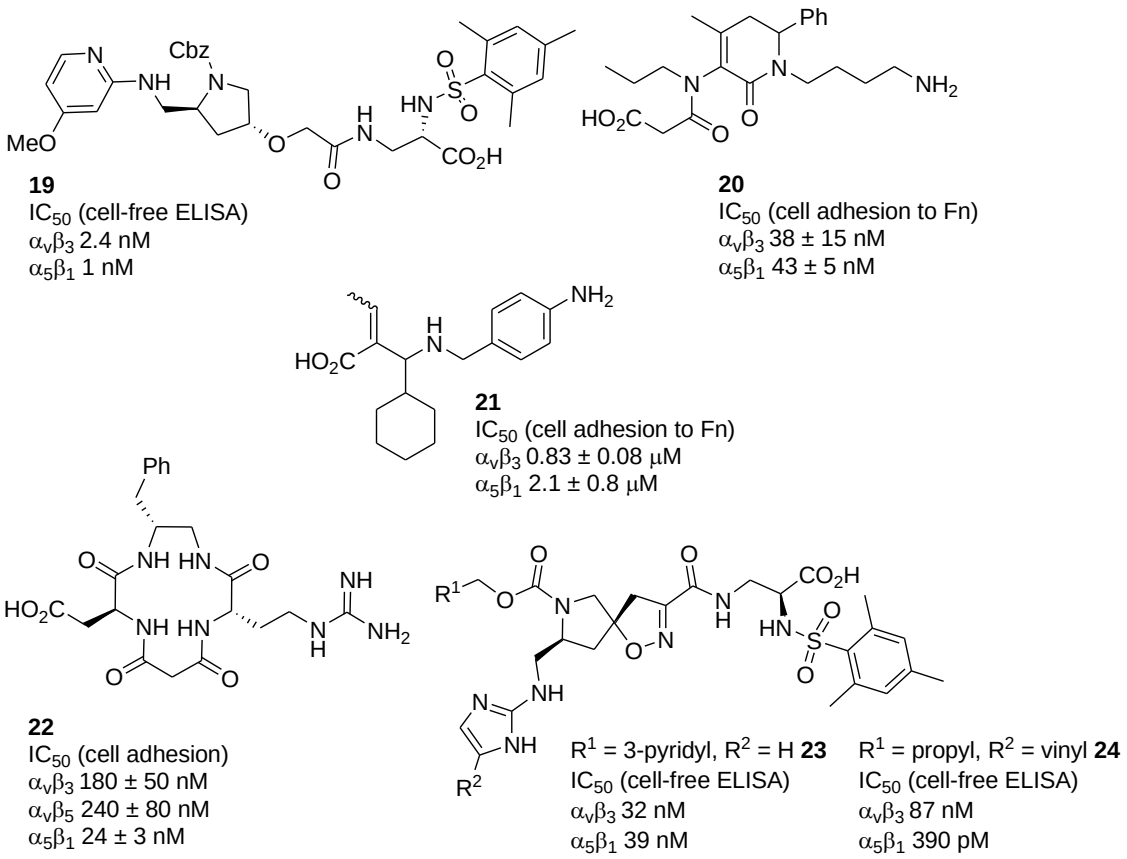


Figure 7 $\alpha_v\beta_3/\alpha_5\beta_1$ antagonists

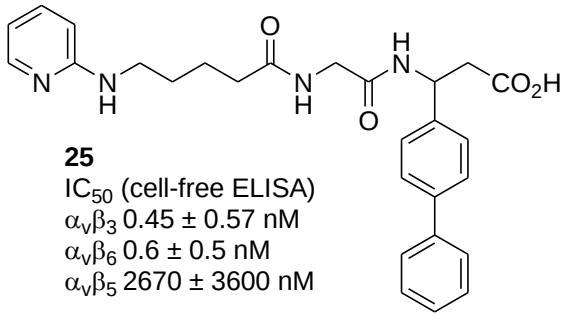
Pyrrolidine derivatives eg **19** (Figure 7) with sulfonamides on the diaminopropanoate moiety proved to be dual $\alpha_v\beta_3/\alpha_5\beta_1$ antagonists with moderate to good selectivity over $\alpha_v\beta_5$. Increasing the lipophilicity of the sidechains increased anti-integrin activity non-selectively, whereas switching from sulfonamide to carbamate gave $\alpha_5\beta_1$ selective antagonists which was rationalized by the greater flexibility of the sulfonamide group allowing it to adopt conformations to fit both integrins' binding pockets.[102]

Dihydropyridinone antagonists were developed to provide more efficient antiangiogenic agents. These molecules lack the exosite binding motif seen in many RGD mimetic antagonists, and also contain very simple arginine mimetics. A primary amine was better than a guanidine group for activity on both receptors, and the most active compound **20** contained a simple butylamine chain.[103] Dehydro β-amino acid **21** with a similar primary amines also showed dual antagonist activity.[104]

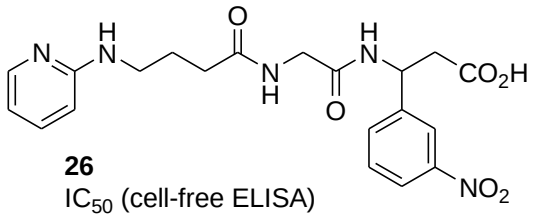
Tetrapeptide mimetic **22** was also designed as an anti-angiogenic agent. A number of stereoisomers were effective in reducing the adhesion of cancer cells with similar levels of affinity for $\alpha_v\beta_3$, $\alpha_5\beta_1$ and $\alpha_v\beta_5$, however only those containing *S*-aspartic acid inhibited human vascular endothelial cell tube formation *in vitro*.[105]

Modifications of isoxazoline compounds to increase potency by introducing a spirocyclic linker gave selective or dual active $\alpha_v\beta_3/\alpha_5\beta_1$ antagonists eg **23**. Selectivity could be changed to favor $\alpha_5\beta_1$ by introducing a carbamate group to the portion of the molecule interacting with the α-subunits.[106] A related molecule **24** was used as a dual targeting agent to selectively deliver nanoparticles to tumors. Dual targeting was more effective at reducing angiogenesis than nanoparticles targeting $\alpha_v\beta_3$ alone which may be due to increased drug delivery when binding 2 receptors or to targeting a wider population of tumor and/or endothelial cells.[107]

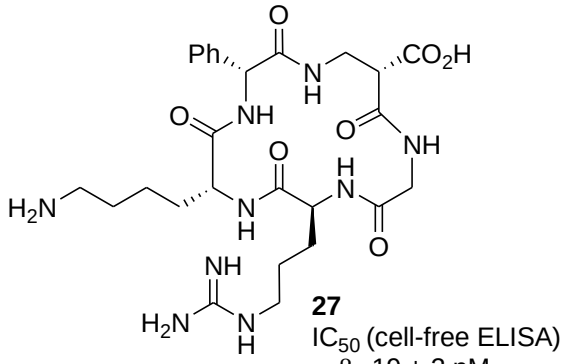
$\alpha_v\beta_6/\alpha_v\beta_3$ and $\alpha_v\beta_6/\alpha_5\beta_1$



25
IC_{50} (cell-free ELISA)
$\alpha_v\beta_3$ 0.45 ± 0.57 nM
$\alpha_v\beta_6$ 0.6 ± 0.5 nM
$\alpha_v\beta_5$ 2670 ± 3600 nM



26
IC_{50} (cell-free ELISA)
$\alpha_v\beta_3$ 13 ± 15 nM
$\alpha_v\beta_6$ 4 ± 4 nM
$\alpha_v\beta_5$ 42 ± 43 nM



27
IC_{50} (cell-free ELISA)
$\alpha_v\beta_6$ 19 ± 2 nM
$\alpha_5\beta_1$ 8.7± 0.7 nM

Figure 8 Dual $\alpha_v\beta_6$ antagonists

$\alpha_v\beta_6$ is upregulated during inflammation and cancer progression, particularly in colon, head and neck and pancreatic cancers.[108] It shares with $\alpha_v\beta_3$ the ability to localize and activate matrix metalloproteinases (MMPs) and activate transforming growth factor-β1 (TGF-β1). There are few examples of small molecules targeting $\alpha_v\beta_6$; the first selective antagonist was developed alongside dual and tri-integrin antagonists **25** and **26** (Figure 8). Sterically demanding aromatic sidechains on the aspartate sidechain mimetic reduce affinity for $\alpha_v\beta_5$ while increasing it for $\alpha_v\beta_6$.[109] The cell-based adhesion assays used in this study provide a useful illustration of experimental design to selectively measure inhibition of a single integrin using a cell line expressing several RGD-binding integrins.

A cyclic peptide **27** with high affinity for $\alpha_v\beta_6$ and $\alpha_5\beta_1$ yet completely inactive against $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_5$ has recently been developed.[9] Docking with a homology model of $\alpha_v\beta_6$ suggested that the high affinity for $\alpha_v\beta_6$ and selectivity over $\alpha_v\beta_3$ is due to D-phenylglycine occupying a hydrophobic binding pocket in the β_6 subunit which is larger than the corresponding pocket in β_3 thus directing the lysine sidechain to forms a salt bridge to the β_6 subunit

Multi-integrin antagonism

The majority of cell types express a range of integrins from several subfamilies on their surfaces. It is extremely unlikely that a single antagonist could affect the entire family of integrin receptors due to significant differences in structures of both α and β subunits. The most obvious difference is the presence or absence of an I-domain in the α subunit controlling the site and mode of ligand binding, but differences in structure of the cytoplasmic domains such as the long tail present in β_4 also modulate integrin function and the binding of cell-penetrating molecules. Despite the differences in structure, it is possible to develop an antagonist with similar affinities for most or all members of a particular subfamily.

Recent development of rabbit monoclonal antibodies suitable for use in formalin-fixed paraffin-embedded tissue samples has paved the way for extensive studies of the expression of the α_v integrin subfamily in primary tumors and metastases, with a particular focus on brain tumors.[67] $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_8$ are frequently coexpressed on gliomas, and increased gene expression is associated with decreased survival; $\alpha_v\beta_5$ and $\alpha_v\beta_8$ were more common in glioblastomas compared to less aggressive tumor types.[110] Evaluation of integrin expression in primary tumors and metastases to the brain revealed α_v integrins were invariably present on tumors and upregulated in brain metastases with the specific heterodimers involved depending on the tumor type; $\alpha_v\beta_5$ is present in most primary tumors, $\alpha_v\beta_3$ and $\alpha_v\beta_8$ are

upregulated in metastases compared to primary tumors. The relative levels of $\alpha_v\beta_6$ in primary tumor and metastasis vary between studies.[111,112]

Based on these results, several suggestions have been made regarding appropriate combination of integrins to target; $\alpha_v\beta_3/\alpha_v\beta_5$ antagonists will target blood vessels but not tumor cells and metastases which often express $\alpha_v\beta_6$ and $\alpha_v\beta_8$ as well as $\alpha_v\beta_3$. $\alpha_v\beta_6/\alpha_v\beta_8$ antagonists may control primary tumor growth and metastasis without normal tissue toxicity, but $\alpha_v\beta_8$ has not been as extensively studied as the other integrins thus its role in tumorigenesis requires further investigation. Overall, the range of integrins expressed on tumors suggests pan-α_v antagonism will be required for efficient prevention and treatment of metastases to the brain since it is the only approach that can target both tumors and the supporting stroma and endothelial cells.[111,112]

Pan-α_v antagonism should be broadly applicable in the treatment of metastasis. α_v signalling has been shown to be highly significant in prostate tumor progression and dissemination. Knockdown of α_v inhibited the growth of $\alpha_v\beta_1/\alpha_v\beta_5$ expressing prostate cancer xenografts in bone through interfering with tumor-microenvironment interactions,[113] and reduced orthotopic tumor growth and bone metastasis initiated by intracardiac injection of prostate cancer stem cells.[114] Antibodies and small molecules targeting α_v have demonstrated antimetastatic effects in a number of tumor types and are reviewed below.

The expression of $\alpha_v\beta_1$ on tumors and metastases has not yet been investigated, and the studies described above do not consider the contribution of non-α_v RGD-binding integrins to tumor progression and metastasis. $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ respectively support angiogenesis and hematogenous metastasis, and can both promote tumor growth, thus should also be considered when designing multi-targeted antagonists.

Anti-α_v antibodies

Antibodies binding all α_v integrins have progressed to clinical trials. Intetumumab (CNTO95), an anti-$\alpha_v\beta_1/\alpha_v\beta_3/\alpha_v\beta_5/\alpha_v\beta_6$ antibody developed to target a wide range of cancer types, was effective at reducing or preventing tumor growth in preclinical models through both tumor specific effects on $\alpha_v\beta_3/\alpha_v\beta_5$, and more clinically relevant action on integrins on both the tumor and surrounding cells.[115] It also diminished hematogenous metastasis to the brain and lungs by reducing the ability of cells to migrate and invade from the vasculature to the metastatic site.[116,117] The anti-metastatic effect was independent of treatment timing, but treatment at the time of removing the primary tumor has been suggested to prevent the formation of metastases from tumor cells released by surgery.[116] Importantly for the development of further pan-α_v targeted drugs, intetumumab has been shown to be safe in both preclinical and clinical studies, despite binding to α_v integrins in a wide range of normal tissues.[118]

In Phase I trials of intetumumab, partial responses occurred in patients with a number of cancer types including metastatic melanoma, angiosarcoma and ovarian cancer,[119,120] and a larger trial on melanoma showed anecdotal evidence of improved overall survival.[121] Despite encouraging results in a Phase I trial on metastatic prostate cancer, in Phase II, intetumumab did not improve progression free survival, but did reduce expression of biomarkers of bone turnover.[122,123] Although these results seem to refute the assertion that multi-integrin inhibition will be effective clinically they must be treated with caution since both low exposure to intetumumab resulting from unfavorable pharmacokinetics and varying levels of target expression in the patients enrolled have been noted during trials.[121] Use of a small molecule with better penetration and half-life in the body may provide more reliable evidence.

In preclinical studies, the humanised deimmunised anti-α_v antibody DI17E6 prevented the growth of melanoma xenografts in mice through acting on human $\alpha_v\beta_3$ expressed on tumors.[124] DI17E6 was well-tolerated in Phase I trials in healthy subjects, and those with

progressing prostate cancer bone metastasis. Clinical trials in prostate cancer are ongoing after multiple patients on the Phase I trial showed stable disease or partial response.[125,126]

Disintegrins

Snake venom disintegrins are a family of small proteins (typically 45–84 amino acids in length) that inhibit multiple integrins including the α_v subfamily, $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$ and others in the β_1 family. As a result of these wide and variable anti-integrin profiles, disintegrins have become useful as probe molecules (eg echistatin), and starting points for anticancer and antithrombotic drug development; the clinically used anti-$\alpha_{IIb}\beta_3$ cyclopeptide Eptifibatide was developed based on the KGD sequence of the $\alpha_{IIb}\beta_3$ specific disintegrin barbourin.[127,128]

Some recent studies investigating the anticancer effects of disintegrins and other snake venom sourced molecules which bind multiple integrins are summarised in Table 2. The majority of these examples contain the RGD recognition sequence, however this is not a requirement for disintegrin activity. Other related sequences also confer high activity on the RGD-binding subfamily,[129] or promote binding to α_4 and β_1 integrins. Not all disintegrins have been fully characterised for their integrin binding profile; many other examples[130-132] show interesting anti-platelet and anti-tumor effects.

Binding a wider range of integrins than many currently used selective antibodies and small molecules is likely to make disintegrins more efficient in controlling cancer growth and dissemination since they will be able to block multiple redundant adhesion mechanisms and signalling pathways. For example, acurhagin C inhibits growth of melanoma cell lines which are unaffected by the $\alpha_v\beta_3/\alpha_v\beta_5$ antagonist cRGDfV.[133] Many disintegrins inhibit platelet aggregation, and thus tumor-platelet interactions involving $\alpha_{IIb}\beta_3$. They are notably effective at preventing the formation of lung metastases when coinjected intravenously with cancer cells.[131,134,135] Vicrostatin, an echistatin/contortrostatin chimera, is selective for activated

platelets and caused no bleeding side effects *in vivo*, suggesting anti-$\alpha_{IIb}\beta_3$ activity can be safely incorporated into an effective anti-metastatic agent.[136]

The clinical use of disintegrins presents some challenges. Naturally produced supply is limited, expensive and hazardous to collect, whereas recombinant disintegrins may have different properties to the naturally sourced material. The echistatin/contortrostatin chimera vicrostatin can be reliably produced recombinantly, avoiding difficulties in supplying contortrostatin in large quantities,[136] and contortrostatin itself has been produced recombinantly[137] and formulated as liposomes to improve half-life and facilitate intravenous administration.[138]

Preclinical experiments with disintegrins indicate that multi-antagonists of the RGD-binding integrins are effective, and generally safe, anticancer agents. Knowledge of disintegrin structures, specifically the conformation of the loop containing the recognition motif, and the nature of the amino acids flanking the RGD tripeptide will provide inspiration for small molecule development.

Disintegrin	**Integrins bound**	**Recognition sequence**	**Anticancer effects**	**Ref**
Salmosin	$\alpha_{IIb}\beta_3$, α_v subfamily, β_1 subfamily	RGD	Reduces hematogenous metastasis. Blocks cell survival signalling.	134,139
Contortrostatin	$\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$	RGD	Anti-angiogenic. Reduces xenograft growth in bone. Reduces hematogenous metastasis. Synergistic with docetaxel	135,140
Vicrostatin	$\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$	RGD	Anti-angiogenic: disrupts endothelial cell cytoskeleton.	136

			Reduces tumor growth	
Acurhagin-C	αv-subfamily (excluding $\alpha_v\beta_5$), $\alpha_5\beta_1$	ECD	Inhibits melanoma cell proliferation and migration. Synergistic with methotrexate	[133]
PIVL	$\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_1\beta_1$, $\alpha_5\beta_1$	Not determined	Inhibits glioblastoma cell invasion and migration	[141]
Eristostatin	$\alpha_{IIb}\beta_3$, $\alpha_4\beta_1$, α_v subfamily	RGD	Reduces hematogenous metastasis. Affects natural killer cell function and interaction with melanoma cells.	[142]
Viridistatin	$\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, other α_v subfamily	RGD	Inhibits tumor cell invasion and migration. Reduces hematogenous metastasis.	[131]

Table 2 Recent examples of disintegrins with anti-cancer activity

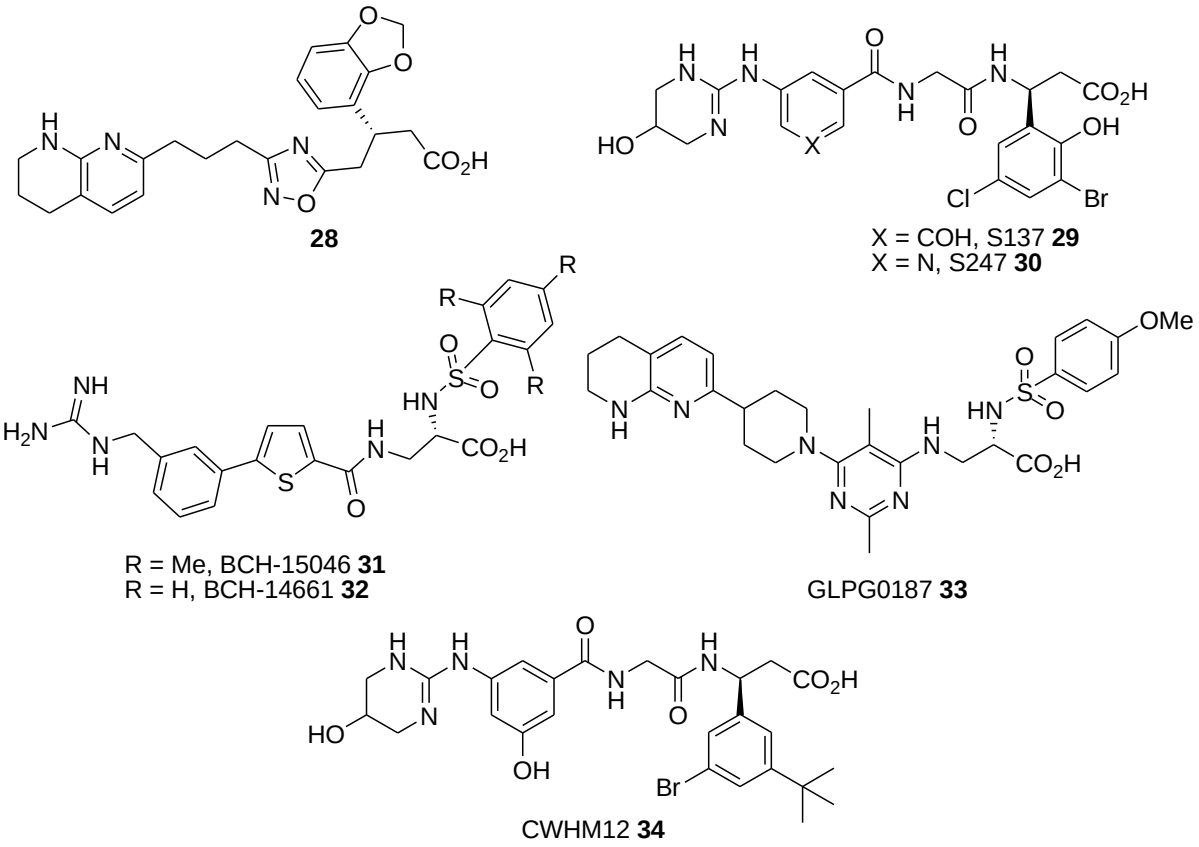


Figure 9 Multi-integrin antagonists

	$\alpha_v\beta_1$	$\alpha_v\beta_3$	$\alpha_v\beta_5$	$\alpha_v\beta_6$	$\alpha_v\beta_8$	$\alpha_5\beta_1$	$\alpha_2\beta_1$	$\alpha_4\beta_7$	$\alpha_{10}\beta_1$	$\alpha_{IIb}\beta_3$	Ref
28	2.6[a]	0.65[a]	1.7[a]	2660[a]	NT	NT	NT	NT	NT	27[b]	[143]
S137 **29**	10.4 ± 7.3[b]	1.6 ± 1[b]	18.1 ± 14.6[b]	11.7 ± 10.7[b]	1.71 ± 0.05[b]	316 ± 228[b]	NT	NT	NT	257 ± 29[b]	[144]
S247 **30**	1.59 ± 0.41[b]	0.40 ± 0.24[b]	1.50 ± 1.26[b]	1.13 ± 0.55[b]	0.65 ± 0.21[b]	64 ± 5[b]	NT	NT	NT	380 ± 92[b]	[144]
BCH-15046 **31**	NT	3.5 ± 0.9[a]	60 ± 50[a]	NT	NT	20 ± 4[a]	NT	NT	NT	0.2[b]	[145]

BCH-14661 **32**	NT	0.033 ± 0.016[a]	72 ± 37[a]	NT	NT	640 ± 256[a]	NT	NT	NT	0.3[b]	145
SC56631 **3**	317[a]	10[b] 120[a]	23[a]	NT	NT	~70000[a]	NT	NT	NT	9[b]	65
GLPG0187 **33**	1.3 ± 0.1[b]	3.7 ± 0.6[b]	2.0 ± 0.6[b]	1.4 ± 0.3[b]	1.2 ± 0.3[b]	7.7 ± 4.0[b]	>10[4,b]	>10[4,b]	NT	>10[5,b]	146
CWHM12 **34**	1.8 ± 0.9[b]	0.8 ± 0.6[a]	61 ± 17[a]	1.5 ± 2.5[a]	0.2 ± 0.1[b]	NR[a]	>5000[b]	NT	>5000[b]	>5000[b]	147

Table 3. IC_{50} (nM) of multi-RGD antagonists in cell-based ([a]) and cell-free ([b]) ELISA. NT = not tested. NR = tested but not reported.

A smaller number of small molecule multi-RGD antagonists have been reported compared with $\alpha_v\beta_3$ or $\alpha_v\beta_3/\alpha_v\beta_5$ targeted agents, although complete testing for selectivity may reveal more; for example screening 'selective' $\alpha_v\beta_3$ antagonists eg. **28** and SC56631 **3** revealed activity on $\alpha_v\beta_1$ as well as $\alpha_v\beta_5$. In this regard the orally bioavailable S137 **29** was described initially as an $\alpha_v\beta_3$ antagonist, but proved to be a pan-α_v antagonist with highest activity against $\alpha_v\beta_3/\alpha_v\beta_8$. Switching to a pyridine ring system gave S247 **30** which has increased affinity for all α_v integrins and measurable activity against $\alpha_5\beta_1$.[144] S247 **30** decreased liver metastasis from established colon cancer splenic xenografts, and continuous infusion reduced lung metastasis from orthotopic implantation of an aggressive breast cancer cell line although it did not reduce growth of the primary tumor in either case.[144,148] In some studies, S247 **30** appeared to exert most effect on the early stages of metastasis,[149] however S137 **29** reduced metastatic tumor burden when administered orally after the primary tumor was removed.[144] Since this mimics the situation of patients with advanced cancer following surgery, it suggests anti-α_v agents will be useful as adjuvant or palliative therapy.

The anti-angiogenic BCH-15046 **31** was shown to be more effective in a range of *in vitro* angiogenesis models than the related $\alpha_v\beta_3$ selective compound BCH-14461 **32** as a result of its ability to prevent both MMP-dependent and collagen-dependent cell proliferation and survival which require $\alpha_5\beta_1$.[145] The high activity of both **31** and **32** against $\alpha_{IIb}\beta_3$ suggests they would show further improved antitumor effects *in vivo*. GLPG0187 is a nanomolar antagonist of 6 integrins developed as a treatment for bone metastasis in breast and prostate cancers;[146,150] its structure has not been reported, but patent analysis suggests **33**. In preclinical studies, GLPG0187 has been shown to be a potent anti-angiogenic and anti-osteoporotic agent, effective in reducing both new and established bone metastases.[151,152] It was shown to be safe and effective at reducing biomarkers of bone turnover in a Phase I trial in healthy volunteers, and has now progressed into trials in patients with advanced cancers. An isopropyl malonate prodrug to increase oral bioavailability is also being investigated.[153] CWHM12 **34** is a nanomolar antagonist of all α_v integrins structurally related to S137 **29**. CWHM12 **34** has not been investigated in cancer, but was effective in preventing and treating renal and pulmonary fibrosis.[147] Cilengitide was not effective here, suggesting that blockade of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ alone is not sufficient to have therapeutic effect in fibrosis perhaps because it leaves other α_v integrins able to activate TGF-β1. The effects of subtle differences in integrin affinity between GLPG0187 and CWHM12 **34**, most notably on $\alpha_v\beta_5$ and $\alpha_5\beta_1$, remain to be investigated.

Discussion

Despite the large number of integrin antagonists reported in the medicinal chemistry literature, a very small number of agents have progressed successfully into the clinic. Multiple hypotheses have been proposed to explain the lack of efficacy seen in clinical trials of agents such as Cilengitide, ranging from reversibly binding antagonists promoting changes in integrin receptor conformation and signalling at low concentrations to issues of selectivity between cell types. In particular, lack of selectivity between the members of an integrin

subfamily has been suggested as a safety issue complicating clinical applications of targeted therapy; to overcome this, the use of highly selective small molecules to provide a tightly aimed targeting of a single integrin expressed by a particular cancer has been proposed.[86]

The use of small molecules targeted to specific integrins as carriers may be useful in delivering cytotoxic agents to cancer cells. However, it does not directly target the integrin mediated processes which are key to tumor proliferation and dissemination, will require the development of a large number of individual targeting agents, and is still liable to failure as cancer cells alter their integrin expression in response to changes in the surroundings or to drug treatment. In contrast, a single small molecule multi-integrin antagonist will provide an anticancer agent applicable to a wide range of tumors which is not vulnerable to the development of resistance or paradoxical effects by changes in expression of particular members of an integrin subfamily. Studies using disintegrins and anti-α_v antibodies have shown that multi-integrin antagonism is likely to be both safe and effective; the ability to bind integrins on multiple cell types does not cause unacceptable normal tissue toxicities, and should result in improved anticancer effects through inhibiting a wide range of tumor and stromal cell interactions with the microenvironment. Further work is required to determine the optimum combination(s) of integrins that should be targeted by an efficient multi-antagonist. A number of the RGD-binding subfamily, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_6$, are already well-established anticancer targets. The biological functions of others, such as $\alpha_v\beta_8$ and $\alpha_8\beta_1$, still largely remain to be defined. Both α_v and β_1 are widely expressed in cells, and the $\alpha_v\beta_1$ heterodimer may become more important as other related receptors are inhibited. Antagonism of $\alpha_{IIb}\beta_3$ is frequently a cause for concern due to its involvement in platelet aggregation and hence the possibility of vascular bleeding as a serious side effect. Early selective $\alpha_{IIb}\beta_3$ antagonists did have a poor safety profile; however, it is noteworthy that the few small molecule dual $\alpha_{IIb}\beta_3/\alpha_v\beta_3$ antagonists investigated to date do not cause prolongation of bleeding time or related side effects. That accepted, the potential benefit of $\alpha_{IIb}\beta_3/\alpha_v\beta_3$

antagonism on preventing life-threatening tumor associated thrombus embolism should not be underestimated. Given the great potential of such compounds in combating tumor dissemination, it is important that development continues and $\alpha_{IIb}\beta_3$ is considered for inclusion as part of the target profile for multi-integrin antagonists.

The medicinal chemistry literature contains a vast number of integrin antagonists. Most of these are selective and designed as antagonists of a single receptor. However, their selectivity has usually been determined against only 2 or 3 related integrins. Full screening of compounds against an entire subfamily is required to determine whether an antagonist is genuinely selective or an unrecognised dual or multi-antagonist. This knowledge will improve the interpretation of biological results obtained using integrin antagonists as probe molecules, and provide structure-activity data to assist with the design of further antagonists with defined selectivity. Combined with full analysis of the integrin expression profile in different diseases, and how it changes in response to drug treatments, such investigations will provide lead molecules not just for cancer, but also other diseases involving angiogenesis, atherosclerosis, fibrosis, and viral infections.

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Abbreviations: ECM extracellular matrix; ELISA enzyme-linked immunosorbent assay; MMP matrix metalloproteinase; VEGF vascular endothelial growth factor.

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Laurence H Patterson has over 30 years' experience in pharma and academia undertaking small molecule drug discovery and mechanisms of action. He has been employed at Fisons Pharma (now AZ), School of Pharmacy, DeMontfort University and The School of Pharmacy, University of London. He is a founding director of Biostatus Ltd and a founding shareholder of Incanthera Ltd. In his present role as Director of the Institute of Cancer Therapeutics he has instigated and oversees a portfolio of drug discovery projects focused on tumor selective chemotherapy and antimetastatic agents. He earned his BSc (hons) at Hatfield Polytechnic/Atomic Energy Research Establishment, Harwell, Oxon and PhD at Chelsea School of Pharmacy, King's College, London with John Gorrod.

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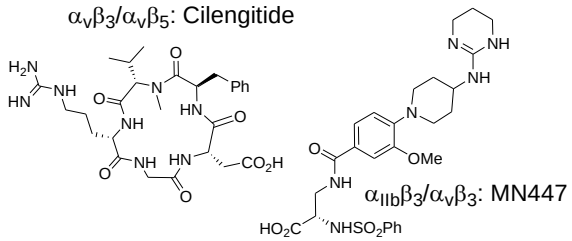

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