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Review

Integrins in glioblastoma: Still an attractive target?

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ABSTRACT

Integrin-mediated signaling pathways have been found to promote the invasiveness and survival of glioma cells by modifying the brain microenvironment to support the formation of the tumoral niche. A variety of cells in the niche express integrin receptors, including tumor-associated macrophages, fibroblasts, endothelial cells and pericytes. In particular, RGD-binding integrins have been demonstrated to have an important role in the epithelial-mesenchymal transition process, considered the first step in the infiltration of tissue by cancer cells and molecular markers of which have been found in glioma cells. In simultaneous research, Small Molecule Integrin Antagonists (SMIA) yielded initially promising results in in vitro and in vivo studies, leading to clinical trials to test their safety and efficacy in combination with other anticancer drugs in the treatment of several tumor types. The initially high expectations, especially because of their antiangiogenic activity, which appeared to be a winning strategy against GBM, were not confirmed and this cast serious doubts on the real benefits to be gained from the use of SMIA for the treatment of cancer in humans.

In this review, we provide an overview of recent findings concerning the functional roles of integrins, especially RGD-binding integrins, in the processes related to glioma cells survival and brain tissue infiltration. These findings disclose a new scenario in which recently developed SMIA might become useful tools to hinder glioblastoma cell dissemination.

Contents

1. Glioblastoma and integrins: state of the art

Glioblastoma (GBM) is the most frequent primary brain tumor in adults. It has a very aggressive course and the few therapeutic options available – neurosurgery, radiotherapy and methylating agents like temozolomide, – have failed. The targeted therapy approach has brought new hope of finding more effective strategies to increase the life expectancy of GBM patients. Molecular pathways involved in the progression of other cancer types have been studied in the hope of finding effective targets but the results have not produced relevant benefits for patients. The rapid proliferation rate and highly infiltrative behaviour of GBM still pose insuperable obstacles to the success of these therapies.

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In recent years, a rare fraction of self-renewing, multipotent tumor-initiating cells, referred to as GBM stem cells (GSCs), supposed to play a crucial role in tumor initiation and maintenance, has been identified and characterized by several groups [1,2]. Key properties of GSCs include the ability to self-renew and to sustain tumor growth in vivo, together with an elevated resistance to chemo- and radiotherapy. This latter feature may partially explain the limited therapeutic efficacy of conventional approaches, which target the bulk population of neoplastic cells but spare surviving GSCs, which are able to regenerate the tumoral mass [3–5].

Studies dealing with the interactions between tumor cells and the extracellular environment focus on the identification of molecules involved in tumor cell proliferation and motility control that would make suitable therapeutic targets. Discussion of the role of integrins in cancer growth, metastatic spreading and neoangiogenesis has been going on in the literature for more than a decade and integrin antagonists, particularly Small Molecule Integrin Antagonists (SMIA), have been studied as new potential weapons against cancer.

Integrins are a large family of cell adhesion receptors composed of two types of transmembrane glycoproteins, α and β, which interact with several extra-cellular matrix (ECM) components to regulate a plethora of cellular effects [6] (Fig. 1).

A number of different integrin subtypes that recognize the tripeptide sequence Arg-Gly-Asp (RGD) found in many ECM proteins, are widely expressed in several cancer types. Among these, the pro-angiogenic αvβ3 is abundantly expressed in high grade brain tumors [7]. Notably, and of particular importance in the context of this review, several reports have demonstrated that some RGD-binding integrins like αv and α6, known to be overexpressed in high grade gliomas compared to non-tumoral tissue or to lower grade gliomas [8], are also expressed in cells forming the glioma niche [9].

These RGD-binding integrins have been studied for their role in tumor progression and metastasis because their expression varies during the transition from a non-neoplastic to a neoplastic state, suggesting that alterations in the adhesion properties of cancer cells may be involved in the early steps of metastasis formation [10].

Driven by biological studies, chemical research has found the field of integrin antagonists particularly attractive and a number of families of interesting molecules have seen the light following the synthesis in 1995 of the prototype Cilengitide [11], opening new roads to research in the field of GBM and cancer in general.

2. Integrins in EMT

Invasion is a key step in the progression towards a malignant phenotype, and occurs when tumor cells translocate from the relatively constrained initial neoplastic mass into neighboring tissues. To accomplish this, cancer cells must somehow detach from the primary tumor and migrate through surrounding tissues, infiltrate through the basal membrane of blood or lymphatic vessel, travel via the circulatory system, and finally colonize distant sites where metastatic foci can be formed.

The process known as endothelial to mesenchymal transition (EMT) promotes the earliest steps of solid tumor invasion and metastasis, and contributes to the conversion of tumors from low-grade to high-grade malignancy. During EMT, epithelial cells undergo a developmental switch that results in decreased adhesion and loss of cell polarity together with increased proliferation, motility and invasiveness. These changes are associated with the down-regulation of epithelial cell surface markers and cytoskeleton components (e.g., E-cadherin, zonula occludens (ZO)-1, claudins, occludins and cytokeratins) and the up-regulation of mesenchymal markers (e.g., vimentin and α-smooth muscle actin) and extracellular matrix components (e.g., collagens and fibronectin) [12].

Among the mechanisms responsible for induction of EMT, the Transforming Growth Factor-β (TGFβ)-dependent signaling pathway plays a pivotal role by modulating invasiveness, angiogenesis, immune evasion and stem cell maintenance [13]. Glioma malignancy and progression are also linked to TGFβ signalling, which plays an important role in GSC survival [14,15]. Quite interestingly,

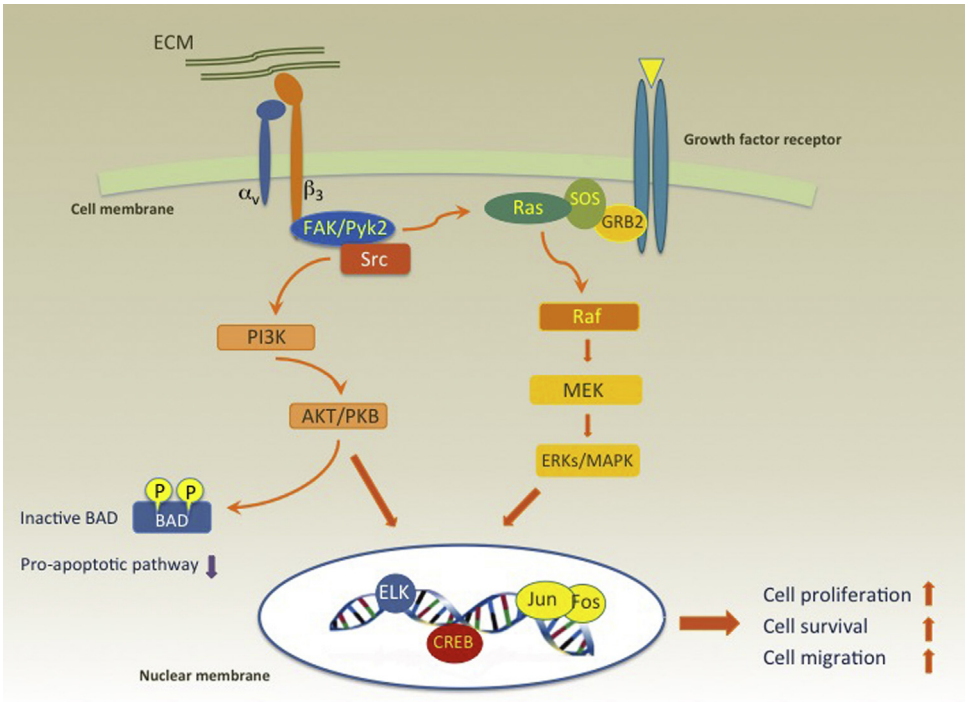

Fig. 1. RGD-binding integrins signalling pathways.

several data indicate that integrins play a key role in TGFβ-dependent induction of EMT.

TGFβ is first secreted in an inactive form in a complex with two proteins – LAP (Latency Associated Peptide) and LTBP (Latent TGF-β-Binding Protein). Its activation, which requires dissociation from the complex, occurs at low pH or through the action of reactive oxygen species, proteases, thrombospondin 1 or several integrins as a result of cell traction forces [16]. Several RGD-binding integrins, including all αv integrins, interact with the LAP/TGFβ1 complex by binding with the RGD motif present in the LAP peptide [17,18]. The traction exerted by integrins bound to the LAP-RGD motif breaks the latency complex, eventually releasing the active form of TGFβ which, in turn, binds to its receptors on the cell surface (Fig. 2).

Upon binding, TGFβ activates a membrane receptor serine/threonine kinase complex that phosphorylates various Smad family proteins. Once inside the nucleus, these Smads act to induce the transcription of a wide variety of TGFβ-related genes. Through this pathway, TGFβ induces the expression of genes involved in the regulation of the cell cycle and the extracellular matrix, like plasminogen activator inhibitor (PAI)-1 together with platelet-derived growth factor receptor (PDGF)-B and downstream tyrosine kinase effectors including PKB/Akt and extracellular regulated kinases (ERKs) [19,20].

Though GBM is not classified as a tumor of epithelial origin, an EMT-like process has been identified in GBM cells and EMT-related genes have been proposed as potential therapeutic targets [21] in GBM therapy. Phosphorylated Smad2 levels have also been proposed as a negative prognostic marker in GBM [20]. The role of TGFβ in GBM, especially in recurrent GBM, has been highlighted in a recent, comprehensive study: the authors found LTBP4 mutations in 11% of patients with recurrent GBM and silencing LTBP4 in two GBM cell lines led to a decrease in the TGFβ-induced proliferation rate [22]. This evidence confirms that the TGFβ pathway is a suitable target for the reduction of GBM recurrence and invasiveness, as previously suggested in other papers [23,24].

3. Integrins in the tumoral niche

Numerous hypotheses have been formulated about the origin of GSCs, e.g. transformation from normal neural stem cell progenitors or de-differentiation of mature glia cells with the acquisition of stemness properties [25]. GSCs are localized in close contact with the vasculature, in the subventricular zone, where non-tumoral neural stem cells also grow [26]. They occur in perivascular or perinecrotic niches, express stemness antigens, and show the same genetic alterations as primary tumors.

Several studies have shown that the hypoxic niche has a role in the maintenance of GSCs, and that hypoxia directly promotes the spread of GSCs through HIF-1α and HIF-2α [26]. Perivascular niches may be described as simple structures, formed by endothelial cells associated with Nestin$^+$ and CD133$^+$ cells, which condition angiogenesis and tumor growth, or as more complicated niches that include endothelial cells, tumor-associated fibroblasts, macrophages, pericytes, microglia and astrocytes with complicated cross-talk between them.

In Section 3.1 we will discuss studies that have investigated the expression, and in some cases the functional role, of integrins among the cells forming the glioma niche.

3.1. Tumor associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs)

Recent findings have opened a new scenario for RGD-binding integrins in GBM. Abundant macrophage infiltration is a common feature of GBMs and an inverse correlation between Tumor Associated Macrophage (TAM) infiltration and GBM prognosis has been reported [27]. TAMs display no phagocytic activity in GBM and recent studies suggest that they may promote GBM tumor progression by secreting cytokines or by promoting neo-vascularization. GSC niches are enriched with TAMs and a correlation between TAM re-infiltration and tumor recurrence has been detected in GBMs

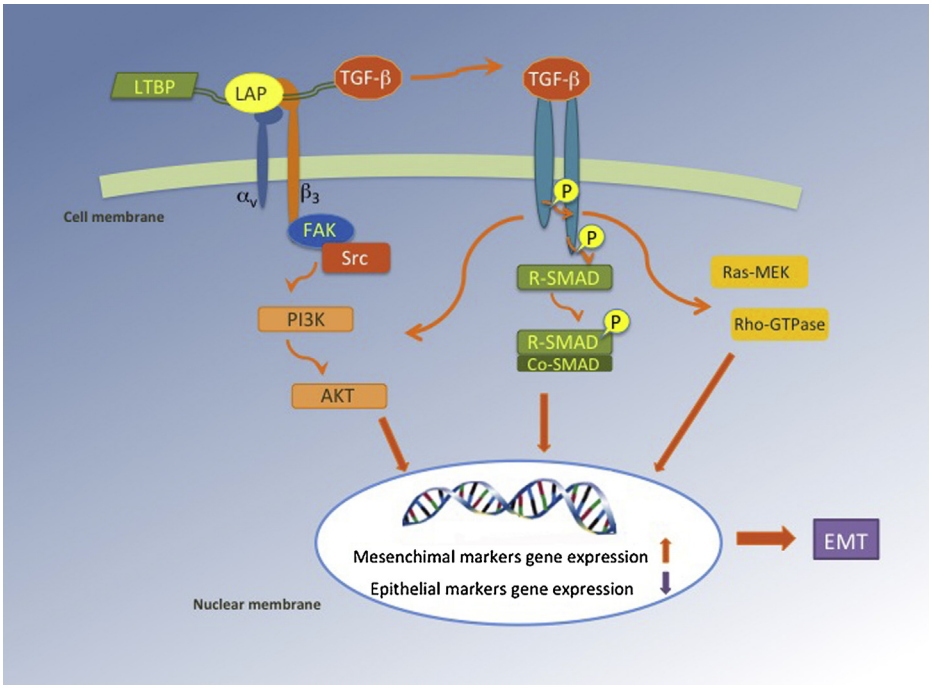

Fig. 2. Integrin-mediated TGFβ receptor activation.

[28]. Taken together these pieces of evidence strongly suggest that there exists an interplay between GSCs and TAMs.

The hypothesis that a chemoattractant secreted into the perivascular niche by GSCs could recruit TAMs was recently confirmed in an interesting work in which periostin (POSTN) was identified among the proteins secreted by GSCs as that most responsible for TAM recruitment in the niche [29]. POSTN is a cell adhesion protein [30] belonging to the family of fasciclin proteins which induces the activation of pathways highly involved in cancer cell survival and adhesion, such as Akt/PI3 K, integrin and Wnt-1 [31,32]. In addition, POSTN promotes tumor progression and malignancy through induction of cancer stem cell growth, invasion and metastatic spreading in breast cancer [30–32]. However, in the context of GBM, the molecular pathways linking POSTN and TAMs are not yet fully understood. It has been suggested that the integrin αvβ3 is the main receptor for POSTN in mediating cell migration [33,34]. This assumption is based on the findings that αvβ3 integrin is widely expressed in GBM and that immunofluorescence staining shows it to be expressed in TAMs in GBM xenografts and primary GBM tumours. Notably, the link between POSTN secreted by GSCs and TAMs is further demonstrated by the finding that POSTN secreted by GSCs induces Akt activation in the U937 monocyte-derived macrophage cell line via stimulation of αvβ3 integrin [32].

The involvement of αvβ3 in the relationship between POSTN and TAMs was demonstrated by using an anti-integrin αvβ3 antibody and an inhibitory linear RGD pentapeptide (Arg-Gly-Asp-D-Phe-Lys). Interestingly, GSC-derived tumours in mice treated with the RGD peptide showed a 70% reduction in TAM density compared to those of mice treated with a control peptide [32], meaning a very important refuelling route for tumor growth was blocked.

Recent papers highlight interplay between RGD-binding integrins expressed by glioma cells and cancer-associated fibroblasts (CAFs). In one, CAF-conditioned medium was found to enhance the chemotactic migration of glioma cells and CAFs were identified in GBM samples [35]. In another, the signaling pathway activated by RGD-binding integrin in cancer cells was found to induce PDGF synthesis and release by cancer cells, leading to fibroblast recruitment in tumor stroma [36]. The picture emerging from this evidence is a sort of loop between cancer cells which, through the RGD-binding integrin pathway, release PDGF to recruit CAFs; and CAFs which enhance the invasive features of glioma cells. Blocking integrins by means of SMIA could therefore break this loop.

3.2. The vascular compartment – endothelial cells

The cerebral microvasculature is a key player in the formation of the glioma niche because of its role in regulating and modifying the interactions between ECM proteins and the matrix adhesion receptors in the different cell populations. The architecture of the cerebral microvasculature compartment is formed by the luminal endothelium capillaries, separated from astrocyte end-feet by the basal lamina, in which another cell type – termed pericyte – is embedded [37]. Moving from the vascular section towards the brain parenchyma, a migrating tumor cell has to find its way through a barrier formed by other brain cell types like neurons, microglia, oligodendroglia and astrocytes.

Tumour-associated blood vessels are structurally and biologically distinct from normal vessels and are known to express integrin αvβ3 and other integrins [38]. It is possible that increased expression of integrins αvβ3 and αvβ5 allows angiogenic endothelial cells (EC) to bind RGD-containing ECM proteins. These enforced interactions have been hypothesized to provide survival and infiltration cues for invading endothelial cells committed to forming new vessels [38]. Interestingly, as an alternative to the classical, widely-discussed use of Cilengitide and other RGD antagonists as antiangiogenic agents [39], the RGD-binding integrins expressed in GBM neovasculature provide an appealing target for the delivery and the internalization of RGD modified nanoparticles [40].

3.3. Pericytes

The causes of the abnormalities present in tumor vasculature are not known but pericytes are required for normal microvascular stability and function [41]. Brain pericytes, located on the abluminal wall of blood vessels, are pluripotential cells with stem cell properties. Bidirectional interactions between endothelial cells and pericytes could be modulated by the expression of specific components such as integrins and other adhesion molecules [42]. Recently, pericytes have attracted considerable attention following a report that hyperplasia of these cells is one of the main charateristics of microvascular architecture in malignant glioma. In co-culture experiments, pericytes have been shown to modulate the angiogenic response of endothelial cells to glioma cells [43]. Perhaps the most striking discovery is that in particular conditions, GSCs give rise to tumor endothelium cells, rather than just tumor cells [44,45]. This hypothesis was elegantly confirmed by the recent discovery that GBM stem cells can trans-differentiate into tumor pericytes during the process of angiogenesis [46]. The authors found that GSCs are recruited towards endothelial cells via the SDF-1/CXCR4 axis and are induced to become pericytes predominantly by TGFβ. However, quite surprisingly given these novel findings, very few reports have addressed the expression and the functional role of integrins in brain pericytes. One study using flow cytometry and immunofluorescence showed that cerebral pericytes express high levels of α5 integrin and lower levels of α1, α2, and α6 integrins but it is not known which of these integrins mediates the pericyte proliferation and migration induced by fibronectin, collagen I and TNF-alpha [47].

Future studies are therefore mandatory to explore the expression of RGD-binding integrins, together with their functional role, in the formation and development of the glioma niche.

4. Small molecule integrin antagonists

In view of the roles played by integrins in GBM growth and infiltration, integrin antagonists may be a valuable tool to modulate these processes, at least in combination with other therapeutic strategies in order to slow down the progress of the disease.

The first integrin antagonist to be tested in clinical trials was Cilengitide (EMD 121974), a cyclic pentapeptide belonging to the RGD-peptide family which, upon binding to the integrin β chain, prevents the interaction of integrins with their endogenous ECM ligands [48]. It displays greater antagonistic activity towards RGD-binding integrins in in vitro displacement assays than its linear counterparts [10] and these features, together with its anti-angiogenic properties, prompted clinical trials testing Cilengitide as a possible antiangiogenic therapeutic agent in GBM patients.

However, the promising features of Cilengitide were disconfirmed when the disappointing results from the CENTRIC phase 3 trial were published [49]. This trial was aimed at evaluating the benefit of Cilengitide in conjunction with standard care (radiotherapy with concomitant and adjuvant temozolomide chemotherapy) in patients with newly diagnosed GBM. No difference in the Cilengitide group compared to the control group was observed when overall survival (OS) and progression-free survival (PFS) were measured. Similar results were reported in phase 2 trials in other cancers [50] and, although another trial (CERTO) of Cilengitide in combination with platinum-based chemotherapy for Advanced Non Small Cell Lung Cancer gave encouraging results, its decline was official.

Several factors might have contributed to the disappointing results obtained: firstly, peptidic and, in part, peptidomimetic integrin antagonists are not orally active and, in any case, can undergo proteolitic cleavage. Furthermore, in clinical trials they were used in combination with alkylating agents to assess whether the antiagiogenic activity of RGD antagonists would potentiate the latters cytotoxic activity [51]. Under these conditions, and used against very aggressive cancer types where even cytotoxic drugs do not achieve the expected results, it was unlikely that any relevant potentiating effect of integrin antagonists would be observed.

On the other hand, peptidic and non-peptidic SMIA show a striking ability to inhibit cell migration in vitro. RGD antagonists have been shown to effectively inhibit GBM cell migration and Focal Adhesion Kinase (FAK) phosphorylation [52]. Putting the pieces of the puzzle together, it is possible that new orally active RGD-SMIA with improved stability, administered with appropriate therapeutic timing, not necessarily concomitantly with alkylating agents, may inhibit GSC infiltation and the spread of cancer in brain tissues. Recent in vitro evidence supports this hypothesis and confirms the possibilty of new roles for RGD-SMIA.

4.1. New players

The poor efficacy shown by Cilengitide in the clinical trials prompted efforts aimed at the synthesis of new peptidic and non-peptidic integrin antagonists with a different pattern of binding properties. The new players that have given interesting in vitro results and could reasonably interfere with the described molecular mechanisms in GBM are essentially small molecules. These molecules, administered in combination with other therapeutic agents such as temozolomide are currently under investigation for their anti-angiogenic and anticancer activity [50]. They include integrin antagonists with different pharmacological profiles: selective αvβ3 antagonists, mixed αvβ3 and αvβ5 antagonists, and α5β1 antagonists.

The inhibition of α5β1 integrin receptors by two different selective nonpeptidic ligands (SJ749 and K34c, Fig. 3) was found to induce apoptosis in human GBM cells [53]. SJ749 is a potent, conformationally restricted, α5β1 antagonist which binds to the isolated receptor in the subnanomolar range. Its chemical structure is characterized by the presence of a spirocyclic nucleus [54] carrying a glutamic acid and a pyridine derivative in place of the RGD aspartic acid and arginine residues, respectively.

K34c maintains the same basic pyridine moiety as SJ749, but is based on a tyrosine core which acts simultaneously as the spacer and the acidic terminal of the molecule. This compound comes from rational in silico design exploiting data from extensive Structure-Activity Relationship (SAR) studies and docking experiments [55].

The integrin antagonist 1a-RGD (Fig. 3), is an RGD-based cyclic pseudopentapeptide that binds αvβ3, αvβ5 and α5β1 integrins with preferential affinity in vitro towards αvβ3. This RGD-containing derivative is based on an azabicyclolactam scaffold which can be viewed as a conformationally constrained mimic of the dipeptide Phe-Pro [56]. The presence of an aromatic moiety flanking the RGD sequence, as in the case of Cilengitide d-Phe residue, seems to be essential to engage hydrophobic interactions

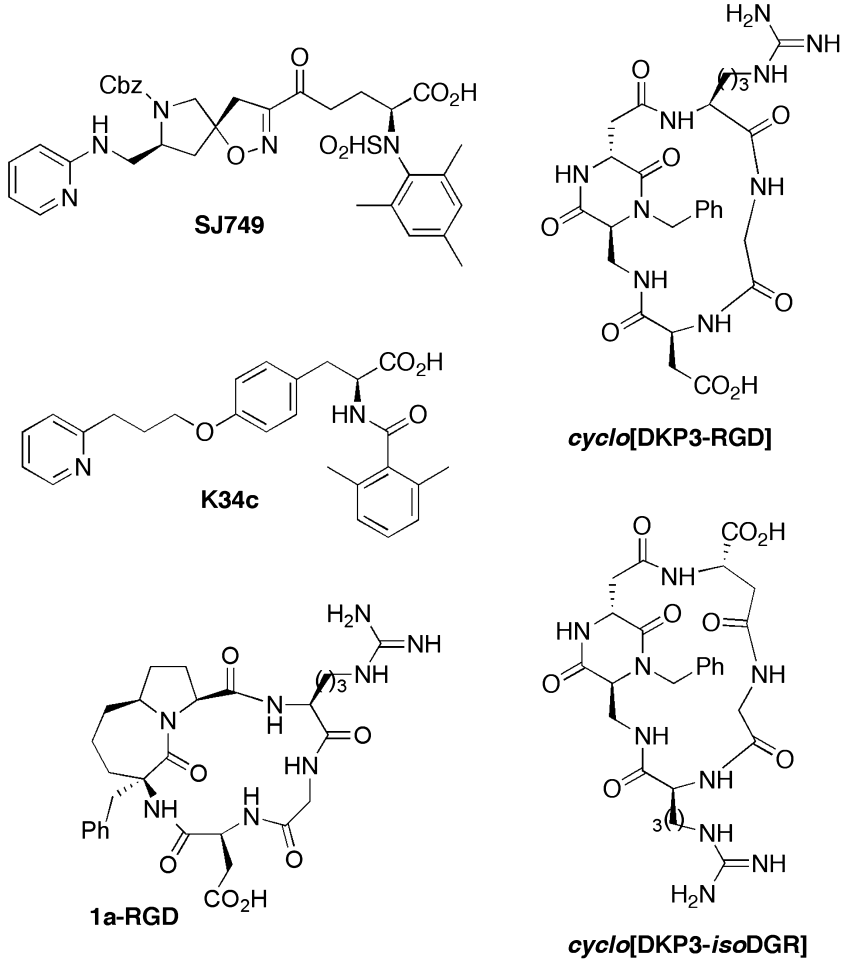

Fig. 3. Chemical structures of Small Molecule Integrin Antagonists (SMIA).

with the αvβ3 binding pocket. Moreover, a recent study reported a central π-π interaction between Trp1496 in the RGD-containing loop of hFN10 (a high-affinity mutant of wtFN10) and Tyr122 in the β3 subunit, which could be exploited in order to design new pure RGD-based antagonists [57]. 1a-RGD proved to be effective in inhibiting RGD-binding-integrin-activated signalling, blocking FAK and Akt phosphorylation and strongly inhibiting cell migration in GBM cells [52]. Interestingly, very similar results were obtained using GSCs isolated from patients, which expressed high levels of αvβ3 and αvβ5 integrins (Paolillo et al., manuscript in preparation).

Recently, a new class of cyclic peptidomimetic RGD-based integrin ligands containing a bifunctional diketopiperazine scaffold (DKP) was reported. The RGD-peptidomimetics derived from DKP scaffolds inhibited biotinylated vitronectin binding to the purified αvβ3 and αvβ5 integrin receptors at nanomolar IC_{50} values. [58].

Recent biochemical studies have also shown that the Asn-Gly-Arg (NGR) motif of the extracellular matrix protein fibronectin can spontaneously change into the *iso*Asp-Gly-Arg (*iso*DGR) sequence by means of a post-translational modification [59]. This asparagine/isoaspartate rearrangement is a well known reaction normally leading to loss of biological activity [60]. In this case, however, the result is a gain in protein function and the formation of a new adhesion binding site for integrins [61]. The *iso*DGR sequence can fit into the RGD-binding pocket of αvβ3 integrin, establishing the same binding pattern as RGD. A recent work [58] reported the synthesis of diketopiperazine-based cyclopentapeptides incorporating the RGD and the *iso*DGR sequences. Among them, cyclo[DKP3-RGD] and cyclo[DKP3-*iso*DGR] (Fig. 3) displayed overlapping inhibitory effects on the FAK/Akt integrin-activated transduction pathway and induced apoptosis in glioma cells after 72 h of treatment.

Another hot new player in the treatment of GBM is GLPG0187, a small molecule integrin antagonist whose chemical structure, probably based on an imidazolidine central nucleus, is still confidential. GLPG0187 blocks the activity of a series of integrin receptor subtypes, including αvβ1, αvβ3, αvβ5, αvβ6 and α5β1. This compound was shown to be effective in the detachment and death of mouse glioma cells [62] and in inhibiting bone homing in a prostate carcinoma model [63]. Phase Ib of the study, which started in March 2011, focused on maximum tolerated dose and biomarker response in patients with solid tumors. Preliminary results confirmed the safety of GLPG0187 and revealed early signs of clinical response in GBM patients. Based on these results and following the request of the investigators, additional patients were included in the study. Unfortunately, at the moment only data concerning safety and tolerability of GLPG0187 in vivo are available.

5. Conclusion

RGD-binding integrins appear to play multiple roles in the GBM niche through several independent mechanisms that regulate a variety of cellular effects, mainly aimed at GSCs survival and migration and which are thus directly responsible for GBM malignancy and recurrence.

Recent studies show new roles for integrins and suggest that, in spite of the disappointing clinical data, RGD-integrins may still be a valid target for new potential drugs, with the necessary *caveat* of not requiring integrin antagonists to do something they cannot: provide a direct antiproliferative effect.

We therefore believe that the development of new high-affinity RGD-binding SMIA with marked anti-infiltrative properties, could open the way to more successful attempts at finding tools to interfere with GBM growth and infiltration.

Conflict of interest

The authors acknowledge no conflict of interest.

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