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Single cell tracking assay reveals an opposite effect of selective small non-peptidic α5β1 or αvβ3/β5 integrin antagonists in U87MG glioma cells

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ABSTRACT

Background: Integrins are extracellular matrix receptors involved in several pathologies. Despite homologies between the RGD-binding α5β1 and αvβ3 integrins, selective small antagonists for each heterodimer have been proposed. Herein, we evaluated the effects of such small antagonists in a cellular context, the U87MG cell line, which express both integrins. The aim of the study was to determine if fibronectin-binding integrin antagonists are able to impact on cell adhesion and migration in relationships with their defined affinity and selectivity for α5β1 and αvβ3/β5 purified integrins.
Methods: Small antagonists were either selective for α5β1 integrin, for αvβ3/β5 integrin or non-selective. U87MG cell adhesion was evaluated on fibronectin or vitronectin. Migration assays included wound healing recovery and single cell tracking experiments. U87MG cells stably manipulated for the expression of α5 integrin subunit were used to explore the impact of α5β1 integrin in the biological assays.
Results: U87MG cell adhesion on fibronectin or vitronectin was respectively dependent on α5β1 or αvβ3/β5 integrin. Wound healing migration was dependent on both integrins. However U87MG single cell migration was highly dependent on α5β1 integrin and was inhibited selectively by α5β1 integrin antagonists but increased by αvβ3/β5 integrin antagonists.
Conclusions: We provide a rationale for testing new integrin ligands in a cell-based assay to characterize more directly their potential inhibitory effects on integrin cellular functions.
General significance: Our data highlight a single cell tracking assay as a powerful cell-based test which may help to characterize true functional integrin antagonists that block α5β1 integrin-dependent cell migration.

1. Introduction

Integrins are αβ protein heterodimers whose non-covalent association defines the specificity of adhesion to particular components of the extracellular matrix (ECM). First recognized as adhesion molecules to the ECM, it is now widely acknowledged that they act as true receptors regulating intracellular signaling and cellular responses including migration, proliferation and differentiation [1–4]. Integrin dysfunction is associated to a large panel of pathological processes such as thrombosis, inflammation, angiogenesis, osteoporosis, infectious diseases and cancer [5]. In recent years, integrins have attracted increasing interest for their potential to act as tumor therapeutic targets [6]. Regulating the crosstalk between cells and their surrounding microenvironment, integrins and their natural ligands are particularly relevant in different key aspects of tumors. Depending on the tumor types, the expression of specific integrins differs between tumoral tissue and their corresponding healthy tissues. For example, gene expression profiling of high-grade glioma associated with gene ontology classification revealed that a class of genes consisting of extracellular matrix components and their regulators is often affected in the patient groups that have the worst survival prognostic [7,8]. As such, fibronectin which is overexpressed in glioblastoma versus normal brain [9] belongs to the cluster of genes associated with a more malignant phenotype. The αvβ3/β5 and α5β1 integrins are among the integrins that bind

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fibronectin through its RGD motif. Although both integrins recognize fibronectin, striking differences between α5β1 and αvβ3 integrin functions and signaling pathways have been described in normal and/or tumoral cells [10,11]. The majority of data addressed the question of cell migration. Although αvβ3 integrins were associated with persistent migration through activation of Rac, α5β1 integrins were linked to RhoA activity and random migration [12]. RhoGTPAse signaling is closely linked to endosomal transport and recycling. Studies indicated that αvβ3 integrins suppress the trafficking of receptors that promote cell migration (VEGFR and EGFR for example). Inversely blocking this integrin by cilengitide, an αvβ3 integrin antagonist [13], activated not only receptors recycling back to the plasma membrane but also concomitantly the recycling of α5β1 integrins leading to increased cell migration [14]. Very selective integrin antagonists may thus be proposed not only to characterize the molecular behavior of individual integrins but also for therapeutic purposes.

Despite the high sequence homology between αvβ3 and α5β1 integrins (αv/α5: 53% identity; β3/β1: 55% identity), unique features of each integrin RGD binding domains have been highlighted [15,16] leading to the rational design of selective ligands [17–19]. Primary tests to screen for antagonists are currently always based on the adhesive function of integrins to their ECM ligands. Non-peptidic small RGD-like integrin antagonist affinity and selectivity have been characterized this way [19–22]. IC50 values are, in general, derived from competitive ELISA tests using the immobilized natural integrin ligand (fibronectin or vitronectin) and the soluble purified integrins (α5β1 and αvβ3 integrins respectively). Few data concerning their effects in a complex cellular context (i.e. cells expressing more than one integrin) have been described.

In this study, we aimed to check if fibronectin-binding integrin antagonists are able to impact on cell adhesion and migration in relationships with their defined affinity and selectivity for α5β1 and αvβ3/αvβ5 purified integrins. For this purpose, we used the U87MG glioma cell line, which endogenously expresses both integrins, and six different RGD-like antagonists with selectivity either for α5β1 or αvβ3/β5 integrins or non-selective. Expression level of α5β1 integrin was genetically modulated to confirm its role in the different experimental conditions. Our results demonstrate that tracking single U87MG cell migration is a reliable functional assay allowing a clear discrimination between α5β1 or αvβ3/β5 integrin antagonists even when both integrins are expressed.

2. Material and methods

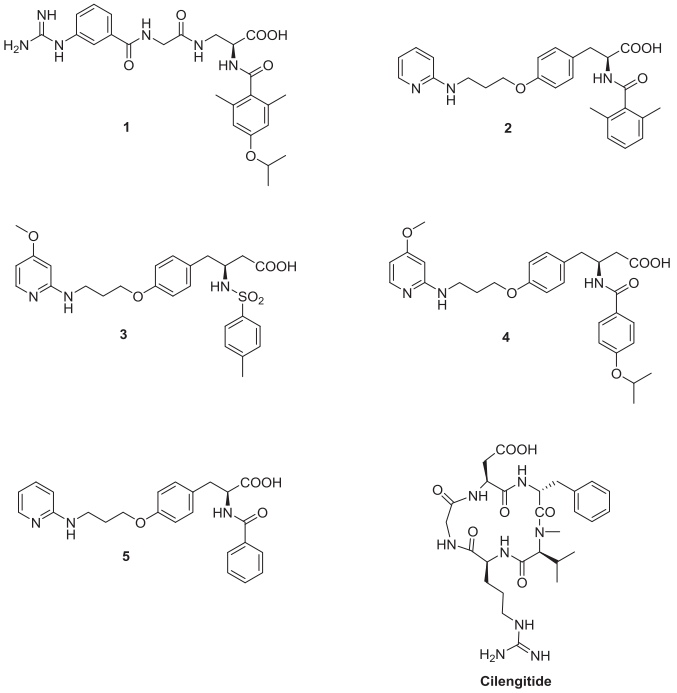
2.1. Drugs and cell lines

Compounds used in the study are described elsewhere and their structures and affinities for α5β1 or αvβ3 integrins are given in Table 1. The compounds 1 and 2 are selective for α5β1 integrin; compounds 3, 4 and cilengitide are selective for αvβ3 integrin and compound 5 has similar affinities for both integrins. The IC50 values of compounds 1 and 3 for the αvβ5, αvβ6 and αvβ8 integrins have been determined according to a previously published method in an ELISA-like solid phase binding assay [21]. Human glioblastoma cell line U87MG was maintained in EMEM (Lonza) supplemented with 10% fetal bovine serum (FBS), 200 IU/mL penicillin/streptomycin and 0.6 mg/mL glutamine, in a 37 °C humidified incubator with 5% CO_2. U87MG cells manipulated to overexpress or repress the α5 integrin subunit have been obtained as described in [7].

2.2. Flow cytometry

Cells were collected with PBS/EDTA (0.53 mM). They were incubated with anti-α5 integrin IIA1 antibody (1:100, BD Bioscience), anti β1 integrin AIIB2 antibody, anti αvβ3 integrin LM609 antibody (10 μg/mL, Millipore) or anti αvβ5 integrin P1F6 antibody (10 μg/mL, Abcam) for 30 min at 4 °C and exposed to Alexa Fluor 488 labeled goat anti-mouse (1:500, Jackson ImmunoResearch Laboratories) at 4 °C for 30 min. A total of 20,000 cells were analyzed using FACS Calibur flow cytometer (Becton–Dickinson, San Diego, USA). The mean fluorescence intensity characterizing surface expression of integrin was measured using the FlowJo Software.

Table 1
Structures and respective affinities of the different compounds for α5β1 and αvβ3 integrins.



Compound	IC50 [nM] α5β1	IC50 [nM] αvβ3	References (ref., compound name)
1	2.3	3000	Heckmann (2008), 44b Rechenmacher (2013), 1
2	3.1	1624	Heckmann (2008), 34c
3	108	0.65	
4	127	0.86	Rechenmacher (2013), 2
5	243	190	Heckmann (2008), 34a
Cilengitide	11	0.2	Rechenmacher (2013) Mas-Moruno (2011)

2.3. Cell adhesion assay

Cells (50,000 cells/well) were plated in the presence of drugs or controls (DMSO 0.2%) on purified fibronectin (gift from Pr Carreira, Cergy Pontoise, France) or vitronectin (gift from Pr Luis, Marseille, France) coated 96-well plates and incubated for 1 h. Adhesion was measured after coloration with crystal violet (0.2% ethanol, w/v) by absorbance recording at 595 nm. Polylysine (Sigma) coated wells were used to measure 100% of adhesion, BSA to define the background. Specific anti-α5 antibody IIA1 (BD Biosciences, France), anti-β1 antibody AIIB2, anti-αv antibody 69.6.5 [23] and anti-αvβ5 antibody P1F6 were used at 10 μg/mL.

2.4. Wound healing assay

Cells (25,000 cells/mL) (1 mL/well) were plated into 24-well ImageLock™ plate (Essen Bioscience). After 24-hour incubation, confluent cell monolayer was scratched with a wound maker™ tool. This system allows having a very precise and repeated imaging within the wound area. Floating cells were washed and cells were incubated in fresh medium containing 10% FBS either with solvent or with drugs,

each treatment performed in duplicate wells. The Incucyte™ Scratch Wound Software was used to capture and analyze wound closure pictures during 20 h (3 images per well and per hour). Wound closure is automatically monitored using relative wound density (% wound closure).

2.5. Single cell tracking

Cells were seeded at low density (500 cells/well) with 1 μg/mL Hoechst 33342 (Sigma) in μClear 96-well black plate (Dutscher, France) in L-15 medium (Sigma) supplemented with 10% FBS.

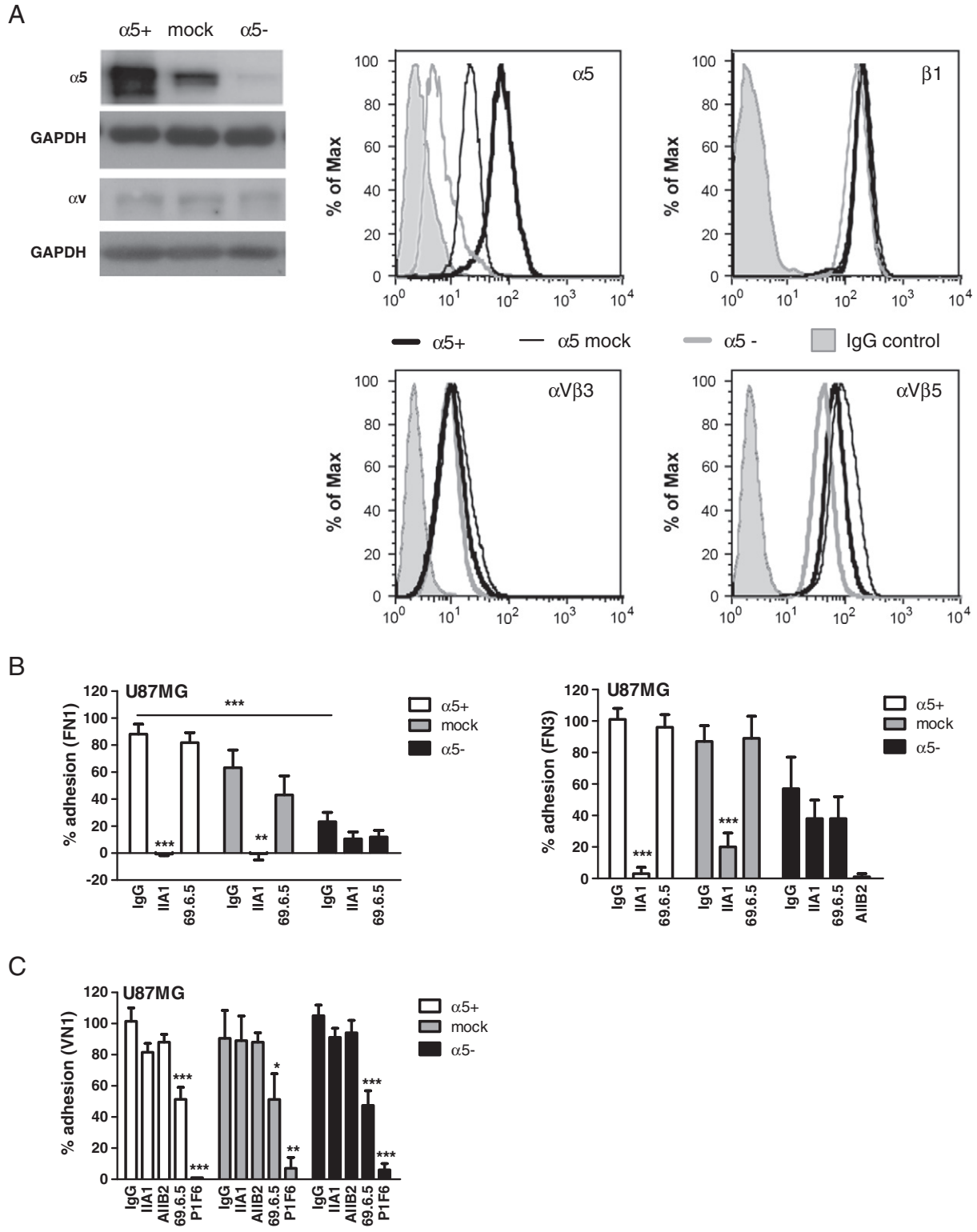


Fig. 1. Respective contributions of α5β1 and αvβ3/β5 integrins in U87MG cell adhesion to fibronectin and vitronectin. (A) U87MG cell line was manipulated to overexpress or repress the α5 integrin subunit. (left) Verification of the α5 and αv integrin subunit expression by western blot. GAPDH was used as the loading control for western blot analysis. (right) Verification of α5 (IIA1 antibody), β1 (AIIB2 antibody), αvβ3 (LM609 antibody) and αvβ5 (P1F6 antibody) integrin expression at the cell membrane by flow cytometry in the manipulated cells. (B) Adhesion of U87MG cells to fibronectin (left: 1 μg/mL; right: 3 μg/mL) in the absence or presence of inhibitory anti-α5 or anti-αv antibodies. (C) Adhesion of U87MG cells to vitronectin (1 μg/mL) in the presence of inhibitory anti-α5 or anti-αv antibodies. Mean ± SEM of 3–4 experiments in triplicate.

Alternatively, wells were coated with human fibronectin (1 μg/mL). After 24 h, medium was removed and fresh medium containing drugs or solvent was added. Migration of cells was followed by fluorescent microscopy (IN Cell Analyzer 1000, GE Healthcare) during 6 h. Analyses were performed only on cells tracked during the entire assay. Speed, persistence and trajectories were computed with Excel software.

2.6. Western blot analyses

Cells were lysed with Laemmli sample buffer (Biorad) on ice and lysate was heated at 90 °C for 10 min. Samples were loaded and run on precast 10% SDS PAGE gels (Biorad) and transferred to PVDF membranes (GE Healthcare). After blocking for 1 h at room temperature, the blots were incubated overnight at 4 °C with specific primary antibody. Primary antibodies used were anti-α5 integrin 1928 1/1000 (Millipore), anti-αV integrin 1930 1/1000 (Millipore), or anti-GAPDH 1/50,000 (Millipore). Membranes were subsequently incubated with a secondary antibody conjugated to horseradish peroxidase 1/10,000 (Promega) and developed to CL-exposure films (Kodak). GAPDH was used as housekeeping protein to serve as the loading control for cell lysate sample.

2.7. Statistical analysis

Data are represented as mean ± SEM. The values were obtained in at least three independent experiments (n). Statistical analyses were done by Student's *t* test with GraphPad Prism program where $p < 0.05$ was considered significant.

3. Results

3.1. Characterization of U87MG adhesion on fibronectin and vitronectin in relation with α5 integrin expression level

U87MG cells express α5β1 integrin endogenously and were manipulated to increase or decrease the expression of the α5 integrin subunit (α5 + and α5 − cells respectively) as confirmed by total protein extract western blot analysis (Fig. 1A, left) or by flow cytometry analysis of membrane-localized integrin (Fig. 1A, right). We also screened the cells for αv integrin and showed that expression level was not changed upon α5 integrin expression manipulation of the cells (Fig. 1A, left). Expression of β1, αvβ3 and αvβ5 integrins at the cell membrane was confirmed by flow cytometry analysis with respectively AIIB2, LM609 and P1F6 specific antibodies (Fig. 1A, right). Adhesion of cells to fibronectin (known to be recognized both by α5β1 and αvβ3/β5 integrins) was first investigated. On 1 μg/mL fibronectin-coated wells, U87MG cell binding to fibronectin was strongly dependent of the α5β1 integrin expression level (Fig. 1B, left). U87MG-α5 − cells showed decreased adhesion compared to the α5 expressing cells with 20% adhesion versus 60% adhesion for U87MG-mock and 100% adhesion for U87MG-α5 + respectively. Adhesion was completely inhibited by the α5β1 integrin specific antibody IIA1 (10 μg/mL) in the two α5β1 integrin expressing cell lines. Conversely, no inhibition of adhesion was denoted in the presence of 69.6.5 antibody (10 μg/mL) which is specific for αv integrin [23]. Similar results were obtained when adhesion of U87MG cells to 3 μg/mL fibronectin-coated wells was examined (Fig. 1B, right) suggesting that αv integrins were not implicated in fibronectin binding of these cells. Although U87MG-α5 − cell adhesion to fibronectin was independent of α5 and αv integrins, the specific anti-β1 integrin antibody AIIB2 inhibited this adhesion suggesting that an αβ1 heterodimer different from α5β1 is implicated in this cell adhesion to fibronectin (Fig. 1B, right). U87MG cells were able to adhere to vitronectin (1 μg/mL), the specific ligand of αv integrins, regardless of their content in α5β1 integrin (Fig. 1C) and this adhesion was partially inhibited by 69.6.5 αv-antibody but not by IIA1 α5-antibody confirming that cell binding to vitronectin used αv integrins. Complete inhibition of binding was obtained by P1F6 antibody specific for αvβ5 integrin (Fig. 1C). These data show that U87MG cell binding to fibronectin is predominantly mediated by α5β1 integrins, whereas binding to vitronectin favors αv integrins involvement. These cells thus appear useful to investigate effects of integrin antagonists with known selectivity for each integrin.

Table 2
Respective affinities of compounds 1, 3 and cilengitide for αV integrins. nd. not determined.

Compound	IC50 [nM] αvβ3	IC50 [nM] αvβ5	IC50 [nM] αvβ6	IC50 [nM] αvβ8
1	>1000	>1000	433 ± 10.1	37 ± 3
3	0.65 ± 0.05	199 ± 21	>1000	>1000
Cilengitide	0.54	10.9	nd	nd

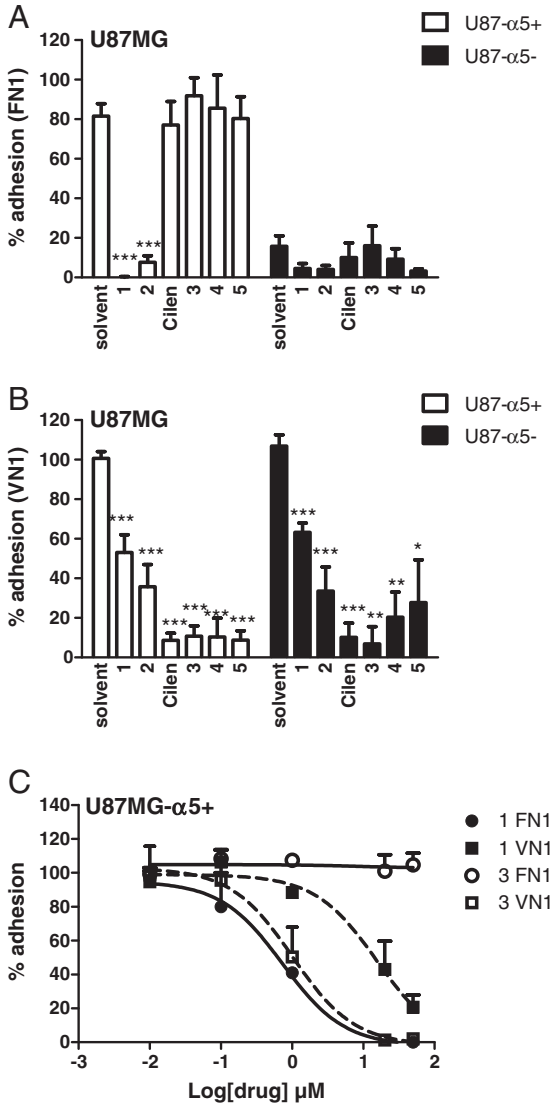


Fig. 2. Inhibition of cell adhesion to fibronectin or vitronectin by integrin antagonists. (A) Adhesion of U87MG cells on fibronectin (1 μg/mL) in the absence or presence of small antagonists (20 μM). (B) Adhesion of U87MG cells on vitronectin (1 μg/mL) in the absence or presence of small antagonists (20 μM). Mean ± SEM of 3–4 experiments in triplicate. (C) Dose–response curves of compound 1 (α5β1 integrin selective) and compound 3 (αvβ3 integrin selective) for the inhibition of U87MG-α5 + cell adhesion on fibronectin (solid lines) or vitronectin (dashed lines).

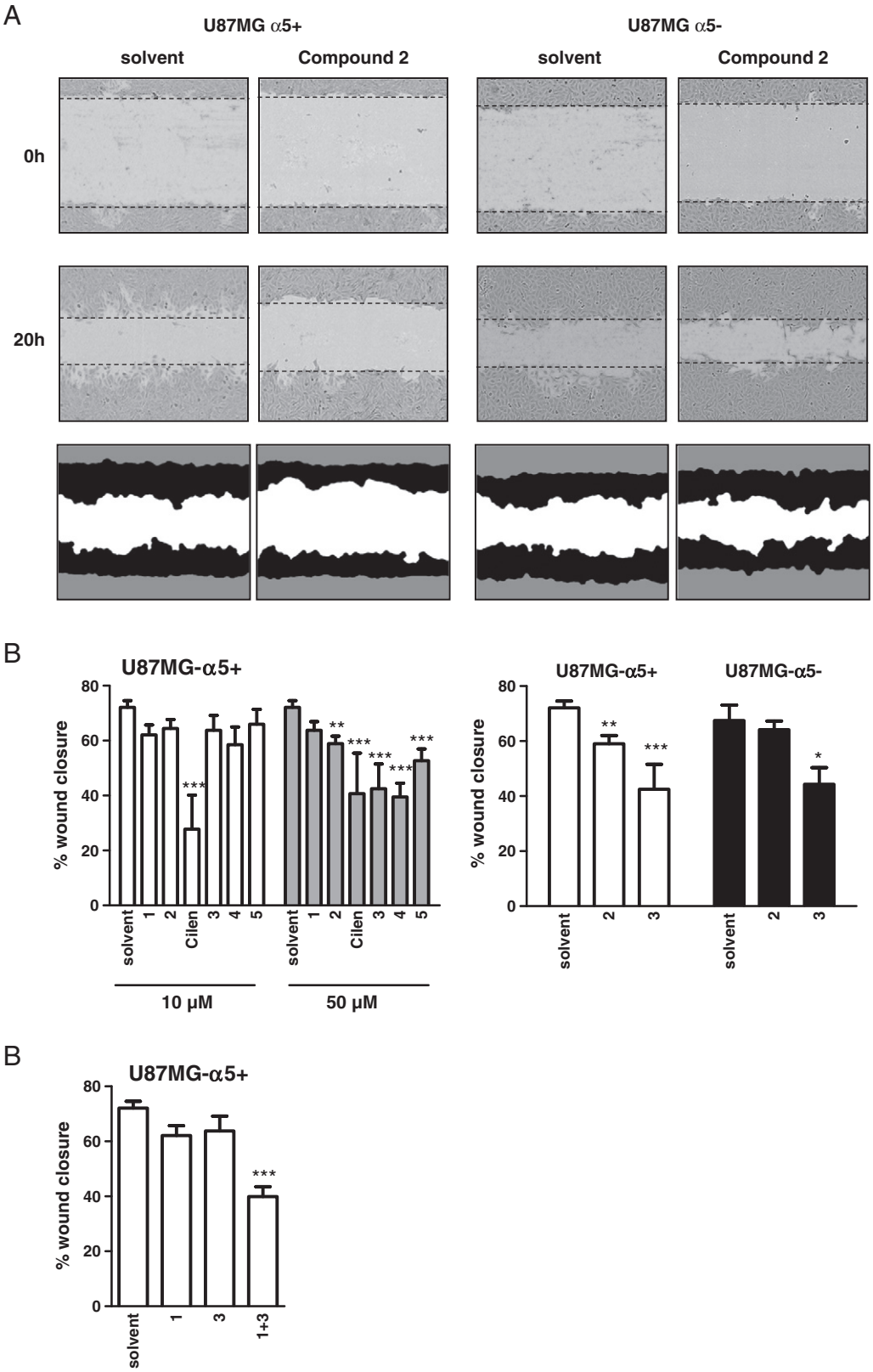


Fig. 3. Effects of integrin antagonists on U87MG cell wound recovery. (A) Representative pictures of U87MG-α5+ (left) and U87MG-α5− (right) cell wound closure at 0 h (top) and 20 h (middle) and schematic representation of the recovered surface (20 h) (bottom). (B) Left. U87MG-α5+ cells were treated either by the solvent or by the different integrin antagonists at 10 or 50 μM. Histograms represent wound closure after 20 h of migration as a percent of wounded area at 0 h. (mean ± SEM of 3–4 independent experiments). (B) Right. Inhibition of wound recovery by compound 2 and compound 3 (50 μM) in U87MG-α5+ or U87MG-α5− cells. (C). U87MG-α5+ cells were treated by compound 1 or compound 3 or both at 10 μM (mean ± SEM of 3–4 independent experiments).

3.2. Effects of integrin antagonists on cell adhesion

We selected several molecules of our series of integrin antagonists to study their effects on glioma cell adhesion. Their affinities for α5β1 and αvβ3 integrins (extrapolated from their IC_{50} inhibitory values calculated from adhesion assays of soluble pure integrin to selected ECM ligands [18]) are shown in Table 1. Two compounds, 1 and 2, highly selective for α5β1 integrin versus αvβ3 integrin were compared to compounds 3, 4 and cilengitide selective for αvβ3 over α5β1 integrin and compound 5 having no selectivity. We also checked the affinities of the most selective compounds 1 and 3 on other αv integrins (Table 2). Compound 1 was unable to bind αvβ5 integrin although compound 3 and cilengitide also recognized this integrin (Table 2). In addition, compound 1 proved able to recognize αvβ6 and αvβ8 integrins with modest affinities although compound 3 did not (Table 2). The α5β1 integrin-dependent binding of U87MG cells to fibronectin was clearly affected by compounds 1 and 2 but not by compounds 3, 4, 5 and cilengitide and this inhibition was dependent on α5 integrin expression (Fig. 2A) as expected from the precedent results. Binding of U87MG on vitronectin was almost totally abrogated by αvβ3/β5 selective ligands (cilengitide and compounds 3 and 4) and compound 5 whatever the level of α5β1 integrin (Fig. 2B). Unexpectedly, compound 1 and 2 also affected cell binding to vitronectin (but less than the other compounds) with no relationship with α5 expression level, suggesting that they may also affect αv integrin when used at high concentrations (20 μM). These data revealed that compounds 1 and 2, at high concentration, may not only affect the α5β1 integrin but also other integrins. Although exhibiting similar affinities for both integrins, compound 5 is more efficient to displace cells from vitronectin suggesting that integrin binding to vitronectin is less strong than binding to fibronectin.

In order to nevertheless confirm the respective selectivity of the drugs either for α5β1 or for αvβ3/β5 integrins, we performed dose–response assays with compounds 1 and 3 on U87MG-α5+ cells. Results were in accordance with our previous data showing that compound 1 and compound 3 respectively were more potent for inhibiting cell binding either to fibronectin (EC50 of 0.4 μM and >50 μM respectively) or to vitronectin (EC50 of 15 μM and 1 μM respectively) (Fig. 2C).

3.3. Effect of integrin antagonists on cell migration (wound-healing assays)

We next investigated whether these integrin antagonists may affect selectively the migration of U87MG glioma cells into a wound generated by scratching a cell monolayer. Wound healing was quantified at different time points after the scratch. After 20 h, the area of the wound recovered by migrating cells was equivalent for all U87MG cells independently of their level of α5β1 integrin expression (Fig. 3A). Data also showed that, at a concentration of 10 μM, only cilengitide was able to inhibit wound recovery of U87MG cells expressing the α5β1 integrin although at higher concentration compounds 2, 3, 4 and 5 also affected cell migration (Fig. 3B, left). Effect of compound 2 on migration was α5 dependent and effect of compound 3 was α5 independent (Fig. 3B, right). Results suggested that both α5β1 and αvβ3/β5 integrins may be involved in wound recovery. To confirm this hypothesis, we compared wound recovery in the presence of either compound 1 or compound 3 at 10 μM (which were shown to be inactive at this concentration — Fig. 3B) to the wound recovery obtained in the presence of both drugs. Treating the cells with both compounds together led to a significant inhibition of wound recovery (Fig. 3C). Data suggest that cell migration involved in the recovery of the wound is dependent on α5β1 and αvβ3 integrins and that simultaneous inhibition of the two integrins is required to get an effect. Our data indicate thus that this migration assay will not be useful to investigate the selectivity of integrin ligands.

3.4. Effect of integrin antagonists on single cell migration

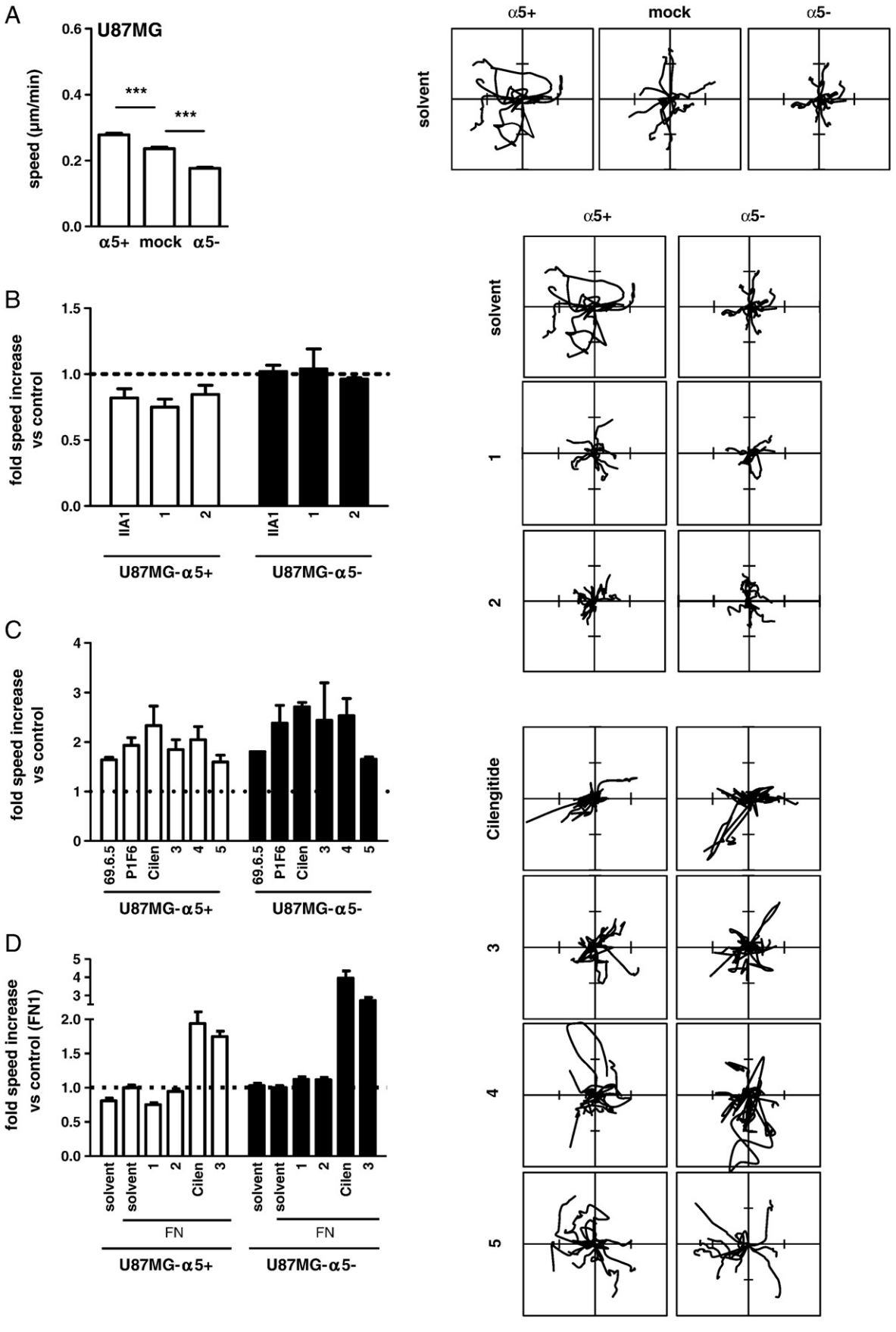
It is already known that single cell migration behaves differently than cell migration into a wound. In this respect the random mobility of U87MG cells was addressed by analyzing cell migration at low cell density and quantifying the speed of migration of individual cells over 6 h in the absence and presence of the antagonists. Diagrams of migratory trajectories of representative cells are shown either in the absence or in the presence of the integrin antagonists (Fig. 4A, B and C, right). Quantitative analysis revealed that speed of migration was dependent on α5β1 integrin expression level in U87MG cells (Fig. 4A, left). Compounds 1 and 2 were able to decrease U87MG-α5+ cell speed and these effects were abolished in U87MG-α5− cells. Similar results were obtained with IIA1 antibodies, further confirming the implication of α5β1 integrin in this process (Fig. 4B, left). Intriguingly, in similar experiments, both cilengitide and compounds 3, 4 and 5 rather increased the speed of migration independently of α5β1 integrin expression level (Fig. 4C, left). This effect appeared αv integrin dependent as 69.6.5 and P1F6 antibodies also enhanced cell speed. These experiments were conducted on non-coated culture wells potentially implicating cell-secreted extracellular matrix and fibronectin from the serum. We wondered if fibronectin coating (1 μg/mL) of the wells may affect these results. Data (Fig. 4D) indicated that fibronectin enhanced U87MG-α5+ cell migration without affecting U87MG-α5− cell migration confirming that only α5β1 integrin is positively implicated in this migration assay. In this experimental setting, αvβ3/β5 integrin antagonists kept their cell speed enhancing properties.

Relationships between drug affinities for αvβ3 integrin and cell migration were next investigated. We plotted the affinities of the different compounds for αvβ3 integrin against the U87MG-α5+ cell speed and we found a slight correlation between these two parameters (Fig. 5A). A stronger significant correlation was obtained in U87MG-α5− cells (Fig. 5A) favoring the hypothesis that α5β1 integrin exerts an opposite effect compared to αvβ3/β5 integrin in this assay. Interestingly, persistence of cell migration (ratio of the vectorial distance to the total distance length traveled by the cell) was clearly decreased only by the αv integrin antagonists (Fig. 5B), an effect which seems increased in U87MG-α5− cells (Fig. 5C).

Data show that U87MG single cell migration is highly related to α5β1 integrin expression, blocked by specific α5β1 integrin antagonists and increased by αvβ3/β5 integrin antagonists. In this assay, selectivity of the antagonists determined in adhesion tests was clearly associated with opposite effects on cell migration even when used at high concentrations. In our experimental conditions, single U87MG cell tracking may be an original cell function-based assay useful to characterize new integrin antagonists.

4. Discussion

Integrins are cell surface receptors largely implicated in migration processes [3,2] and currently explored as pertinent therapeutic targets in particular for cancer [5,6,24]. We addressed in this work the impact of the functional inhibition of α5β1 and αvβ3/β5 RGD-dependent integrins by selective antagonists on U87MG cell adhesion and migration. We demonstrated that the expression level of the α5β1 integrin determines U87MG cell adhesion on fibronectin and migration. Two different modes of glioma cell migration were investigated reflecting, although partially, what may happen in vivo. The wound healing assay induces a collective, directed migration and is widely used to decipher the cell migration parameters [25]. Leader and follower cells coordinate their cytoskeletal activity and mechanical forces through cell–cell interactions implicating adherens junction proteins such as N-cadherin in glioma [26,27] In our experimental conditions, the U87MG cell migration in the wound healing assay was not affected by the expression level of α5 integrin but similarly affected by α5β1 and αvβ3/β5 integrin antagonists. Respective contributions of the different integrins in this


A
U87MG
speed (µm/min)
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U87MG-α5+
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fold speed increase vs control
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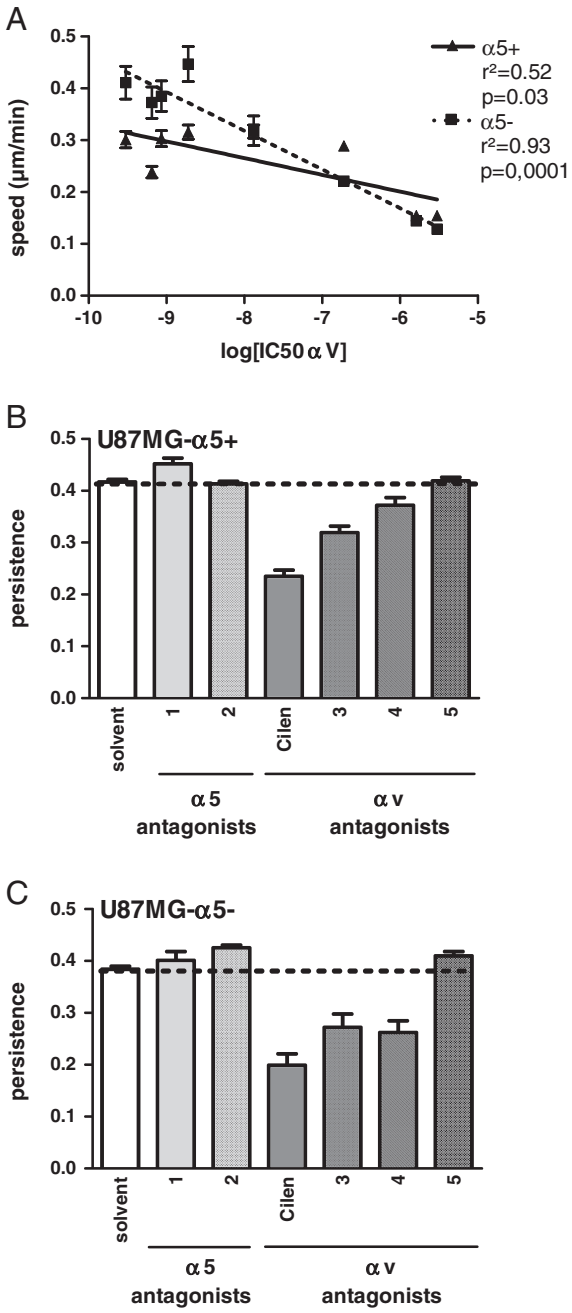

Fig. 5. Relation between affinities of compounds for αvβ3 integrin and cell migration. (A). Linear regression analysis of the cell speed plotted versus the affinities for αvβ3 integrin of the different compounds in U87MG-α5+ cells (solid line) or U87MG-α5− cells (dashed line). (B) (C). Persistence of U87MG cell migration represents the ratio of the vectorial distance to the total distance length traveled by the cells during 6 h. Persistence of U87MG-α5+ (B) or U87MG-α5− (C) cell migration is shown in the presence of solvent or integrin antagonists (mean ± SEM of 3–4 independent experiments with 100 to 400 cells included per experiment).

setting would not be easy to delineate. Single cell migration appears more specifically related to cell-matrix adhesion parameters. By contrast to scratch assay, single cell tracking analysis revealed the U87MG cell line as a clear α5β1 integrin-dependent model in which opposite effects of α5β1 and αvβ3/β5 integrin antagonists have been recorded. We therefore propose that U87MG single cell tracking experiments in 2D may be considered as a first-line function-based assay useful to characterize new selective integrin antagonists.

Integrins have been first considered as proteins only implicated in cell adhesion to extracellular matrix components and the search of antagonists has been built on this characteristic. Hence, the antagonistic affinities for a specific integrin were extrapolated from adhesion inhibition experiments [28]. Indeed, binding affinities were calculated from experiments using either adhesion of purified solubilized integrins on their specific ECM ligands [18,21,29], or adhesion of cells expressing only the integrin of interest [21,30] or alternatively adhesion of cells expressing two integrins with one of them blocked with an antagonist [31,32]. In this study, we used the U87MG glioma cell line expressing both α5β1 and αvβ3/β5 integrins in which only the expression of α5β1 integrin was manipulated. Exploration of integrin antagonist properties in this cellular model may better reflect what could happen in tissues expressing several integrins concomitantly. The experiments indicated that U87MG cells are primarily α5β1 integrin dependent for adhesion to fibronectin and αv integrin dependent for adhesion to vitronectin as confirmed by their relationship to integrin levels and also by their sensitivity to specific antibodies. The αvβ3/β5 integrin antagonists were only able to affect cell binding to vitronectin whatever their affinities for the α5β1 integrin (ranging from 11 to 243 nM; Table 1). Inversely, the α5β1 integrin antagonists at high concentration were able to not only completely inhibit the cell binding to fibronectin but also with fewer efficacies the cell binding to vitronectin even if their affinities for αvβ3/β5 integrin are low (over 1 µM; Table 1). Our data suggest that only drugs with very high affinities for α5β1 integrins may disturb their binding to fibronectin although this is not the case for drugs inhibiting the binding of αvβ3/β5 integrin to vitronectin. Discrepancies may be in part explained by the fact that α5β1 integrin (and not αvβ3 integrin) more strongly adheres to fibronectin due to its recognition of the fibronectin synergy site [33]. Alternatively, integrin crosstalk may be implicated as data showing that β1 integrin inhibitory antibodies negatively modulate αvβ3 integrin binding to vitronectin have been reported [34].

Respective implication of α5β1 and αvβ3/β5 integrins in cell migration has been the subject of numerous studies. Alterations in the expression of fibronectin-binding integrins can strongly affect parameters of cell migration. In the past, collaborative interactions between α5β1 and αvβ3 integrins have been suggested for cell motility [35]. More recent studies point to distinct functions in cell adhesion [10,36], spreading [37] and migration [38,39] through activation of different molecular mechanisms [4,40]. Moreover, it has been shown for αvβ3 integrin that its deletion [41] or its blockade with cilengitide [14,42] increased cell speed through activation of the recycling loop of α5β1 integrin. Accordingly, our data also show that U87MG single cell migration speed was increased in the presence of αvβ3/β5 integrin antagonists but decreased with α5β1 integrin antagonists. In addition, persistence of cell

Fig. 4. Effects of integrin antagonists on U87MG single cell migration. (A) (left) Speed of U87MG cells recorded during 6 h in relation with α5 integrin expression level. Histograms represent the mean ± SEM of 3 independent experiments with 100 to 400 cells included per experiment. (right) Diagrams representing the migrating trajectories covered in 6 h of ten representative U87MG cells. (B) (left) Analysis of U87MG-α5+ or U87MG-α5− single cell migration treated by compounds 1 or 2 selective for α5β1 integrin (50 µM). Histograms represent the ratio of antagonist-treated cell speed/solvent-treated cell speed. As control, inhibitory antibodies specific for α5β1 integrin (IIA1) were also included. (mean ± SEM of 3–4 independent experiments with 100 to 400 cells included per experiment). (right) Diagrams representing the migrating trajectories covered in 6 h of ten representative cells.(C) (left) Analysis of U87MG-α5+ or U87MG-α5− single cell migration treated by compounds 3, 4 and cilengitide, selective for αvβ3 integrin or compound 5, non-selective (50 µM). Histograms represent the ratio of antagonist-treated cell speed/solvent-treated cell speed. As control, inhibitory antibodies specific for αv integrins (69–6–5) or αvβ5 integrins (P1F6) were also included (mean ± SEM of 3–4 independent experiments with 100 to 400 cells included per experiment). (right) Diagrams representing the migrating trajectories covered in 6 h of ten representative cells. (D). Analysis of U87MG-α5+ or U87MG-α5− single cell migration on fibronectin-coated wells treated by compounds 1, 2, 3 and cilengitide. Solvent-treated cell migrating on fibronectin is considered as the control experiment.

migration was decreased when αvβ3/β5 integrin was blocked, which confirmed other works [38,41,42]. However, the observed increase in speed and decrease in persistence by these antagonists did not only depend on α5β1 integrin expression in our models as both persisted in α5β1 integrin-repressed cells. The molecular mechanisms underlying the pro-migratory effects of the αvβ3/β5 integrin antagonists deserve further studies. In particular, comparison of their effects in 2D versus 3D migration will be of interest.

To characterize the respective roles of α5β1 and αvβ3/β5 integrins in physio-pathological settings, the use of specific and selective antagonists is crucial. The human α5β1 and αvβ3/β5 integrins are highly homologous and overall shape or domain organization is very similar [15,16,43–45]. It is thus challenging to propose highly selective α5β1 or αvβ3/β5 integrin small antagonists. We nevertheless achieved this goal with the design of compounds able to selectively recognize distinct integrin subtypes. They are powerful tools to characterize integrin subtype-dependent fundamental processes as shown recently [20,22,46]. In the current work, we extended our investigations on their biological effects in cells expressing both α5β1 and αvβ3/β5 integrins which appeared more complex. Interestingly it appears that although cell adhesion assays could not completely delineate a selective effect of α5β1 integrin antagonists, single cell migration assay demonstrates their clear and significant integrin subtype specific inhibitory activity.

5. Conclusions

Integrin antagonists are nowadays selected on their capability to inhibit the adhesion of purified integrins to their natural ligand. Although this approach has given valuable informations about the affinities and selectivity of antagonists, more complex data may be obtained when they are evaluated in cells expressing several different integrins. Our data suggest not only that selectivity may depend on the relative expression of the integrins under investigation but also that conclusions may depend on the experimental assays used. We provide a rationale for testing new integrin ligands in a cell-based assay (single cell migration assay) to characterize more directly their potential inhibitory effects on integrin cellular functions.

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