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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

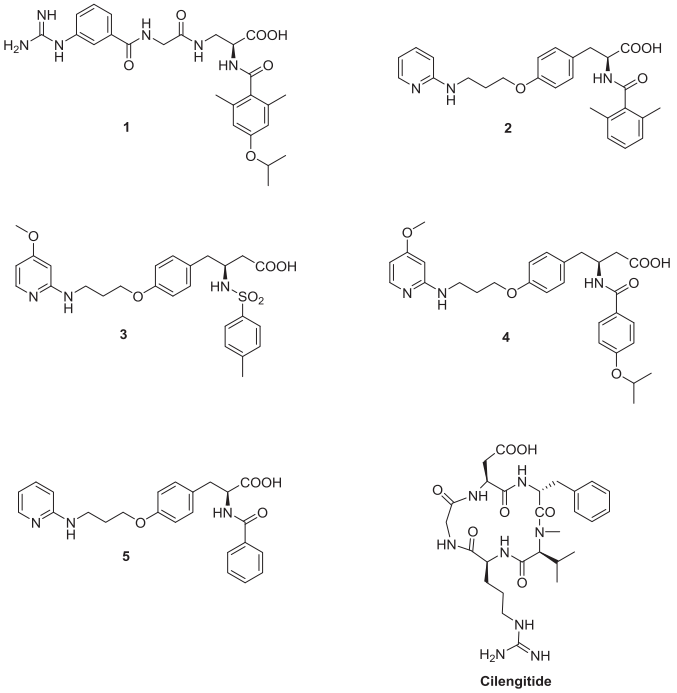
2.1. Drugs and cell lines

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,