European Journal of Medicinal Chemistry 83 (2014) 284–293

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Short communication

Targeting integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ with new β-lactam derivatives

Paola Galletti [a, *], Roberto Soldati [a], Matteo Pori [a], Margherita Durso [a], Alessandra Tolomelli [a], Luca Gentilucci [a], Samantha Deianira Dattoli [b], Monica Baiula [b], Santi Spampinato [b, *], Daria Giacomini [a, *]

[a] Department of Chemistry "G. Ciamician", University of Bologna, Via Selmi 2, 40126 Bologna, Italy
[b] Department of Pharmacy and Biotechnology, University of Bologna, Via Irnerio 48, 40126 Bologna, Italy

ARTICLE INFO

Article history:
Received 8 November 2013
Received in revised form
17 June 2014
Accepted 18 June 2014
Available online 19 June 2014

Keywords:
Lactams
Integrins
Azetidinones
Peptidomimetics
Agonists
Cell adhesion

ABSTRACT

The $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins are widely expressed in different cancer types and recognize the tripeptide Arg-Gly-Asp (RGD) motif present in several extracellular matrix proteins. We report here the design, synthesis and biological activity of some new β-lactam derivatives specifically designed to target integrins. The new molecules contain the azetidinone as the only cyclic framework armed with carboxylic acid and amine terminals spaced from 9 to 14 atoms to switch on recognition by integrins. All tested molecules showed a concentration-dependent enhancement in fibronectin-mediated adhesion of K562 and SK-MEL-24 cells; in particular **1**, expressed a higher affinity towards $\alpha_5\beta_1$ integrin (EC_{50} of 12 nM) and **2** was more selective for integrin $\alpha_v\beta_3$ (EC_{50} of 11 nM).

1. Introduction

Integrins are heterodimeric α/β transmembrane receptors that mediate dynamic adhesive cell–cell and cell–matrix interactions [1–4]. Those receptors are able to sense and respond to different kinds of extracellular cues, including the chemical, physical, and topographical properties of the cell's microenvironment. The most critical chemical signal transmitted via integrins is the specific molecular composition of the extracellular matrix (ECM) [5].

The ability of integrins to bind and associate with various ECM components such as proteins, transmembrane receptors and soluble ligands largely depends on their structural conformation and these distinct conformations are crucial for regulating both inside-out and outside-in signalling [6]. The activation of intracellular signalling pathways controls cell shape, motility, proliferation, survival, and cell-type-specific gene expression.

This capacity of integrins to activate, integrate and distribute information illustrates the potential of these receptors to serve as functional distribution hubs in a bi-directional information transfer [7]. Because of the important roles of integrins and their ligands in development, immune responses, leukocyte traffic, haemostasis, and cancer, their potential as a therapeutic target is now widely recognized [8].

Among the integrin superfamily, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins play a pivotal role in the formation of new blood vessels [9] and are overexpressed on activated endothelial cells in physiological and pathological angiogenesis [10]. The $\alpha_v\beta_3$ integrin is extensively expressed on tumour cells. There are some evidences that this receptor is present in late stage glioblastomas, ovarian carcinoma, melanomas; it also is an endothelial cell marker in breast cancer and regulates melanoma cell proliferation, survival and metastases [11]. The involvement of integrin $\alpha_v\beta_3$ in highly important pathologies induced the development of potential candidates able to inhibit the functions of this receptor. Commonly, these candidates are referred as antagonists, because the final physiological effect is an inhibition of the usual function of $\alpha_v\beta_3$, but the mode of action of these molecules has not been elucidated. This means that they could be full or partial agonists, inverse agonists or antagonists; they probably engage the integrin altering its conformation as response, preventing intramolecular shape change or dragging a conformational equilibrium towards the inactive form of the receptor [12].

The activation of integrin-mediated cell adhesion by antibodies or small molecules has been recently reported: Vanderslice et al.

* Corresponding authors.
E-mail addresses: paola.galletti@unibo.it (P. Galletti), santi.spampinato@unibo.it (S. Spampinato), daria.giacomini@unibo.it (D. Giacomini).

http://dx.doi.org/10.1016/j.ejmech.2014.06.041
0223-5234/

demonstrated that agonists enhanced the effects of stem cell-based therapies by improving cell retention and engraftment [13a]; Yea et al. found that a single agonist antibody against the alpha chains of integrins can induce human stem cells to become dendritic cells [13b]. It was thus recognized that integrin agonists could open up novel opportunities for therapeutics which gain benefits to increase rather than decrease integrin-dependent adhesion. For instance, a significant factor in chemoresistance in melanoma is a loss of integrin-mediated adhesion; in this case, stimulation of integrin signalling by agonists significantly improved the response to chemotherapy [14a]. Gupta et al. have reported that small molecule mediated activation of integrins, rather than inhibition, reduced leukocyte migration, tissue accumulation and inflammatory injury [14b].

Up to now, a large number of peptidic and nonpeptidic ligands for the $\alpha_v\beta_3$ receptor have been developed, which are all related to the minimal recognition motif RGD (Arg-Gly-Asp) present on $\alpha_v\beta_3$ integrin ligands of the ECM such as fibronectin and vitronectin [11]. As an example, cilengitide (Fig. 1), a small RGD-containing cyclic pentapeptide, is currently in clinical phase III for glioblastoma multiform and in phase II for other types of cancers (e.g., prostate cancer) due to its capability to antagonize $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ integrins [15]. Recent research efforts have focused on improving the pharmacological parameters mainly by altering the polarity and rigidity of the scaffold and the nature of the basic moiety. Among several heterocyclic structures able to antagonize integrins, lactam derivatives found their own niche. In Fig. 1 are reported some lactam derivatives active as integrin ligands.

Some of the ligands include the RGD tripeptide framework (in red, **A** [15], **C** [16], and **F** [17]), others still hold the carboxylic acid and guanidine tails **B** [18] and **G** [19], whereas lactams **D** [20] and **E** [21a] demonstrated that a phenylamine portion could successfully replace the arginine residue. It is interesting to note the insertion of a β-lactam ring as a rigid scaffold in cyclopeptides **F** and **G**.

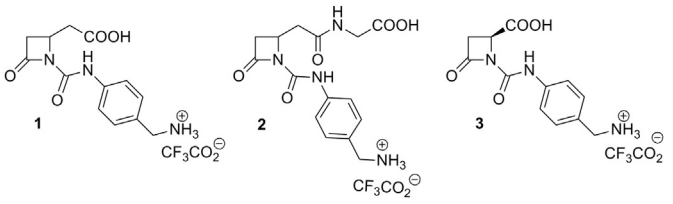


Fig. 2. β-Lactams synthesized and tested.

In previous papers, some of us have identified a series of $\alpha_v\beta_3$/$\alpha_5\beta_1$ integrin ligands with unsaturated β-amino acid fragment [21b]. It was known that incorporation of a distinct β-amino acid into a RGD-containing peptide resulted in the stabilization of specific conformations of the ligand [22]. It was argued that a restricted conformation introduced by unsaturated β-amino acid and of a cyclic structure could give a favourable alignment of both the basic and carboxylate moieties on the ligand, thus meeting the crucial requirements for integrin affinity and selectivity. The β-lactam ring (azetidinone) constitutes *per se* a site of conformational restriction with a β-amino acid moiety in a cyclic structure and constrained features. As a part of our studies on design and synthesis of new β-lactam derivatives [23] and of an on-going interdisciplinary project, we would like to evaluate the ability of azetidinones, not inserted into cyclopeptides, to target integrins. We report the synthesis and the preliminary biological results on K562 (human erythroleukemia expressing $\alpha_5\beta_1$ integrin), SK-MEL-24 (human malignant melanoma expressing $\alpha_v\beta_3$ integrin), and Jurkat E6.1 human T cells (expressing $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrins) cells of some new β-

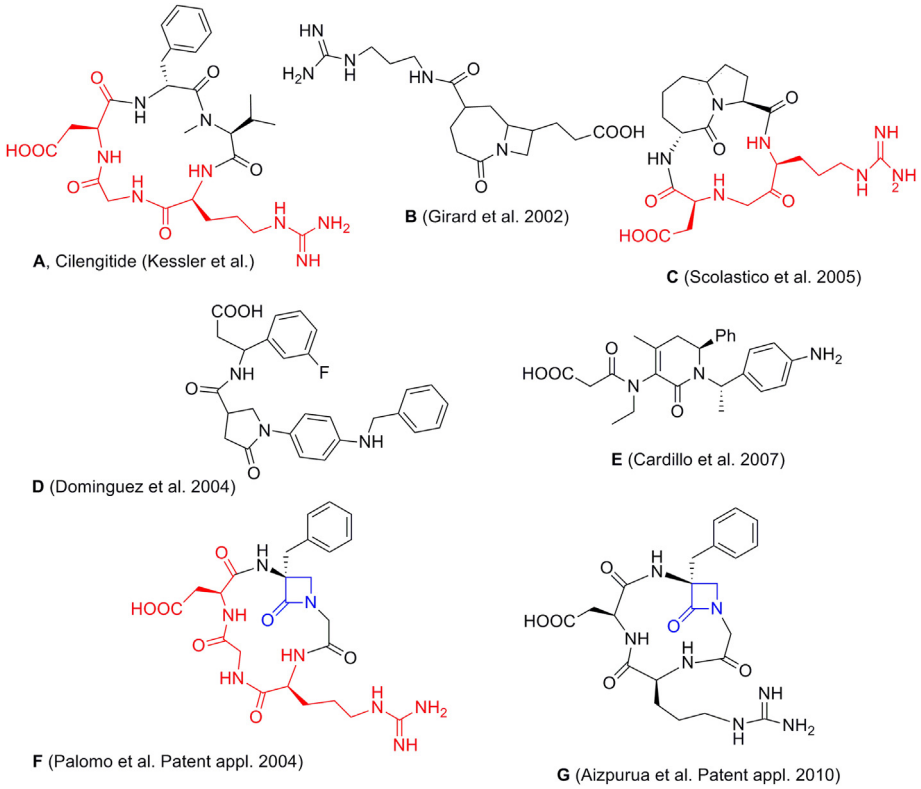


Fig. 1. Cilengitide and lactams as integrin ligands, highlighted in red the RGD sequence and in blue the β-lactam ring. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lactam derivatives. The approach for the design of the new molecules was based on rationalization of known ligands structures: we set out to explore molecules containing the azetidinone as a rigid cyclic framework, armed with carboxylic acid and amine terminus spaced from 9 to 14 atoms to activate a conceivable recognition by integrins (Fig. 2). A 4-amidobenzylamine residue was chosen as the basic terminus directly linked to the β-lactam nitrogen atom thus resulting as imido function. The carboxylic acid was on the C-4 side chain of the azetidinone and differently spaced from the ring: in compound **1** the beta-lactam ring together with the C4 side chain could be considered a sort of beta-glutamic acid derivative, which was elongated with a glycine residue in compound **2**, in the azetidinone **3** the carboxylic acid is directly linked to the beta-lactam ring thus resembling an aspartic cyclic amide. The C-3 position was not substituted in order to mimic the methylene residue of glycine in the RGD peptide. Here we report our discovery of azetidinones as new cell adhesion ligands and their characterization as $\alpha_v\beta_3$ and $\alpha_5\beta_1$ agonists.

2. Results and discussion

2.1. Chemistry

Compounds **1** and **2** were both obtained starting from the commercially available 4-acetoxy-azetidin-2-one. The introduction of the carboxylic function in the C-4 side chain was achieved through a Reformatsky reaction followed by *N*-acylation of the β-lactam ring to give compound **1**. The C-4 side chain was eventually converted into a longer peptidic chain through insertion of a glycine unit to give compound **2** (Fig. 3).

Compound **3** was prepared starting from 4-carboxylic-azetidin-2-one, easily obtained in enantiomerically pure form by ring-closure of an L-aspartic acid diester.

A careful protecting group strategy for the C-4 and N-1 side chains of azetidinones was developed to preserve the β-lactam ring throughout the synthesis, in particular in the final deprotection step, and to enable specific combination of temporary or permanent protecting groups to achieve a full or partial deprotection depending on the synthetic strategy requirements. In Schemes 1–3, the synthetic steps are described in details.

As outlined in Scheme 1, tert-butylbromoacetate and benzylbromoacetate were treated with an excess of metallic Zn in THF to furnish the corresponding Reformatsky reagents which were then coupled with 4-acetoxyazetidin-2-one **4** to give 4-acetate-azetidin-2-one esters **5** and **6** in good overall yields after purification by flash column chromatography (Scheme 1).

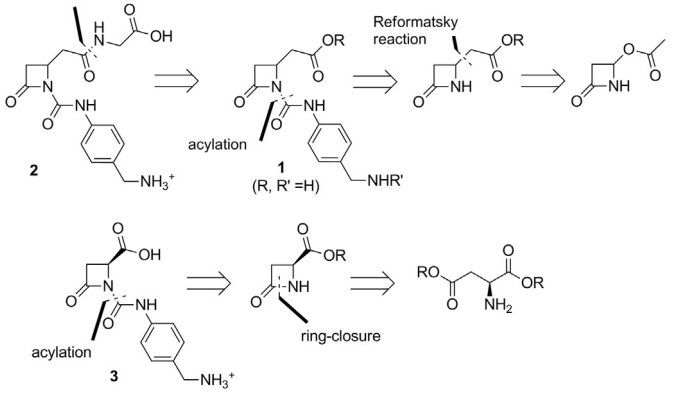
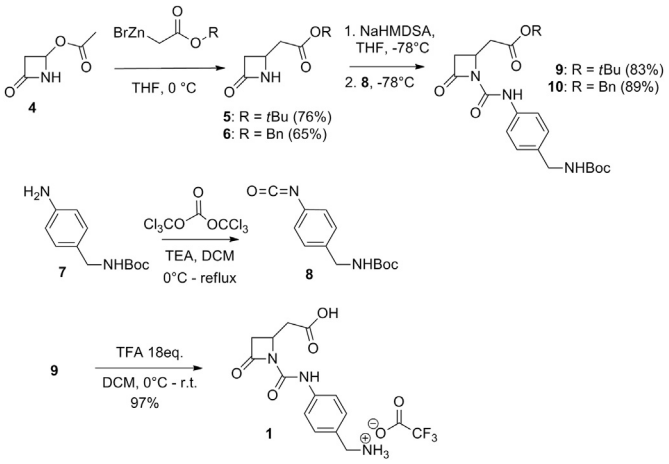
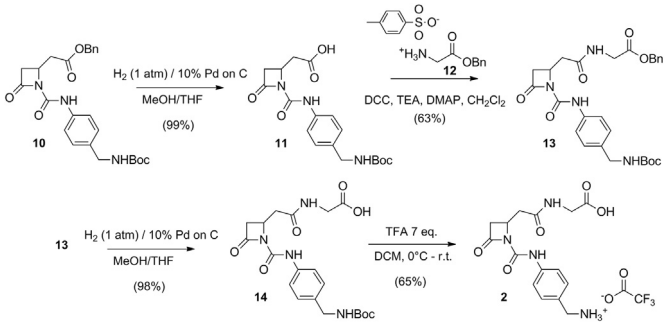


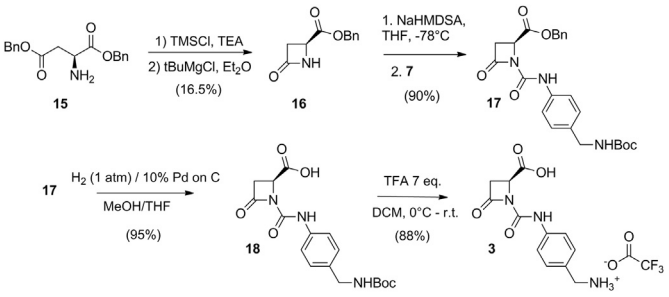
Fig. 3. Synthetic strategy for compounds **1**–**3**.



Scheme 1. Synthesis of compound **1.**



Scheme 2. Synthesis of compound **2**.



Scheme 3. Synthesis of compound **3.**

The N-1-side chain was constructed starting from 4-aminobenzylamine **7** selectively protected with a Boc group on the benzylic amine and then transformed into the corresponding isocyanate **8** with triphosgene. Treatment of azetidinones **5** and **6** with NaHMDSA (Sodium bis(trimethylsilyl)amide) followed by addition of isocyanate **8** furnished compound **9** and **10** in good yields after purification by flash column chromatography. Compound **9** was then deprotected (both C-4 and N-1 side chains) by treatment with trifluoroacetic acid to give compound **1** as trifluoroacetate salt (Scheme 1).

The benzyl ester of compound **10** was in turn selectively deprotected to give acid **11** so that a DCC mediated coupling with benzylglycine **12** could be accomplished to deliver compound **13** (Scheme 2). Compound **13** was subsequently deprotected in a two steps procedure furnishing **14** by hydrogenolysis and then treating

with TFA; azetidinone **2** was successfully isolated as trifluoro acetate salt (Scheme 2).

As to the synthesis of **3**, the starting 4-carboxylic-azetidin-2-one **16** was obtained by a Grignard mediated cyclization of L-aspartic acid dibenzylester **15** (Scheme 3). Enantiomerically pure **16** was then *N*-acylated to give **17** and deprotected in two steps with the same procedures exploited above furnishing **18**, and finally, **3** was isolated as trifluoroacetate salt.

2.2. Pharmacology

The ability of **1**, **2** and **3** to modulate the adhesion of K562 (expressing $\alpha_5\beta_1$ integrin) or SK-MEL-24 (expressing $\alpha_v\beta_3$ integrin) cells to immobilized fibronectin (10 μg/mL) was evaluated. These cell models are widely used to investigate potential ligands capable of influencing cell adhesion mediated by the above mentioned integrins [24].

Interestingly, β-lactams **1**, **2** and **3** showed a concentration-dependent enhancement in fibronectin-mediated adhesion of K562 and SK-MEL-24 cells (Fig. 4). With regards to $\alpha_5\beta_1$ integrin expressing cells, **1** was the most potent in enhancing cell adhesion with an EC_{50} of 12 nM; it was ≈five times less potent *vs* $\alpha_v\beta_3$ integrin. β-Lactam **2** was the most effective toward $\alpha_v\beta_3$ integrin (EC_{50} = 11 nM) and **3** was ≈1000 less effective. These cell models were validated demonstrating that the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ agonist fibronectin produces a concentration-dependent elevation of cell adhesion in SK-MEL-24 and K562 cells (data not shown). Reference antagonists were capable to displace cell adhesion in SK-MEL-24 cell line (Ac-Asp-Arg—Leu-Asp-Ser-OH, IC_{50} = 25 nM) and in K562 cell line (cyclo-Arg-Gly-Asp-D-Phe-Val, IC_{50} = 34.7 μM).

In another set of cell adhesion assays, **1** and **2** were ineffective to modify Jurkat E6.1 cell adhesion mediated by $\alpha_4\beta_1$- (toward vascular cell adhesion molecule-1, VCAM-1) and $\alpha_L\beta_2$- (toward intercellular adhesion molecule-1, ICAM-1) integrin ($IC_{50} \geq 100$ μM, data not shown), thus demonstrating selective interactions by the new beta-lactam ligands.

The ability of the new β-lactams to increase cell adhesion was then tested in the absence of fibronectin. In a second set of experiments, adhesion of K562 and SK-MEL-24 cells to wells previously coated by passive adsorption with the most active β-lactams **1** and **2**, fibronectin as comparison, and BSA as a control, were tested (Fig. 5). Both β-lactams **1** and **2** produced a significant adhesion of

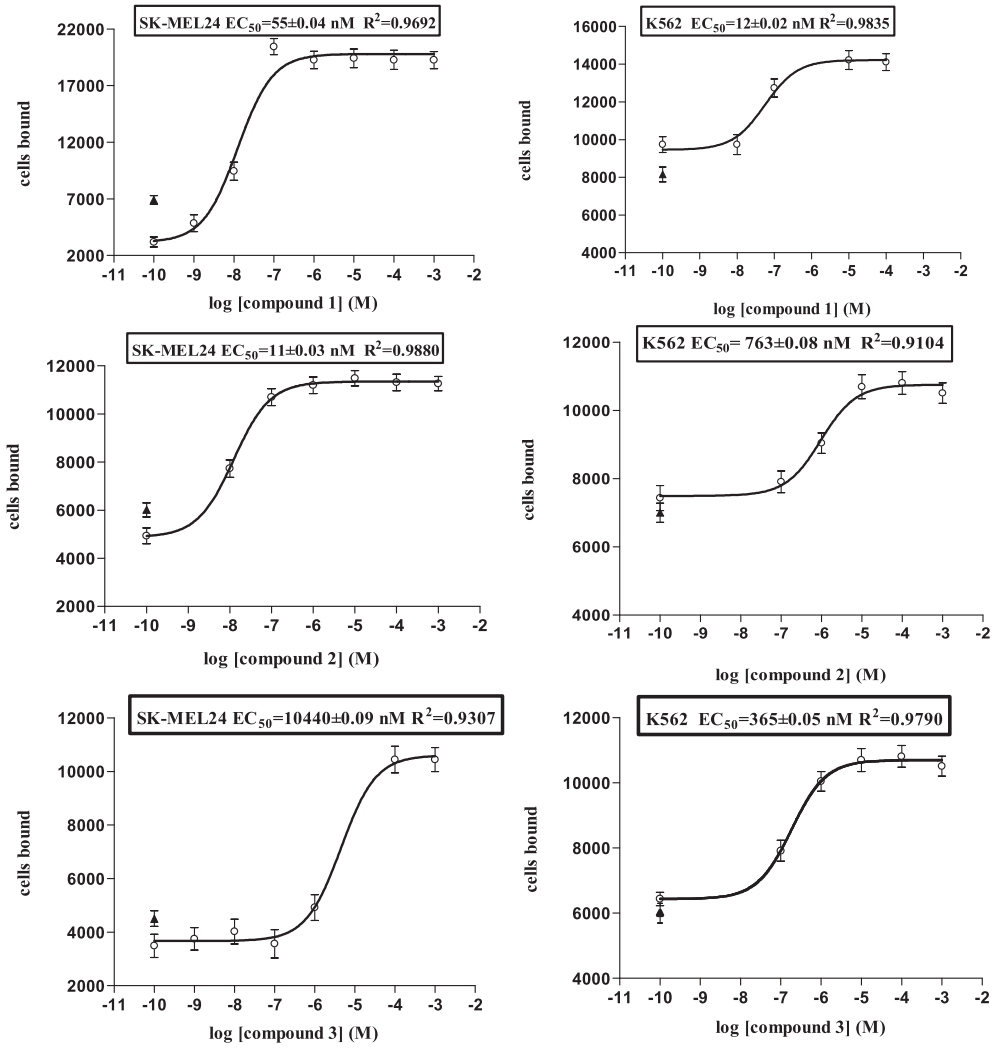


Fig. 4. Compounds **1**, **2** and **3** enhance fibronectin-mediated adhesion to K562 cells (right column, expressing $\alpha_5\beta_1$ integrin) and SK-MEL-24 cells (left column, expressing $\alpha_v\beta_3$ integrin). Concentration–response curves showing the effects of **1**, **2** and **3** on cell adhesion are reported. Cells were incubated for 30 min at room temperature with each compound (○) or with the vehicle (▲) as described in the experimental section. Results are expressed as the number of cells attached ±S.E.M from quadruplicate wells and repeated at least three times.

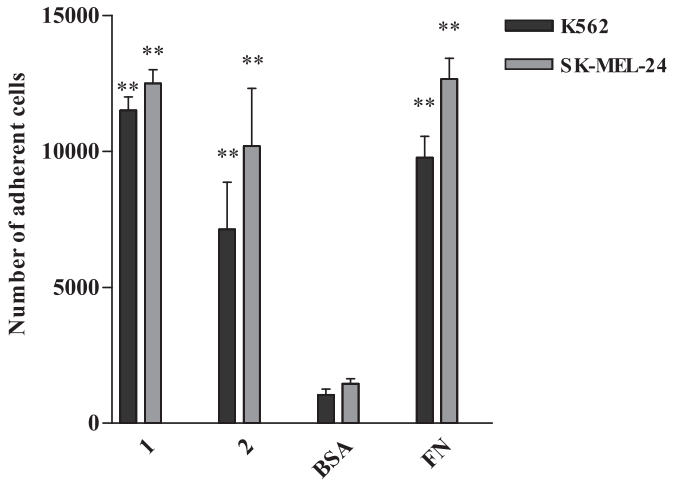


Fig. 5. K562 and SK-MEL-24 cell adhesion to wells coated with 10 μg/mL of fibronectin (FN), compound **1,** or **2** as described under Materials and Methods. Controls were cells plated in wells coated with 10 μg/mL bovine serum albumin (BSA). Each value is the mean ± S.E.M. from four separate experiments carried out in duplicate. $^{**}P < 0.001$ compared to BSA-coated wells (Newman–Keuls test after ANOVA).

K562 and SK-MEL-24 cells, comparable to fibronectin, on the contrary, both cell lines did not adhere to wells coated with bovine serum albumin alone.

Neutralizing antibodies to the β_1 or α_v integrin subunit (10 μg/mL), added to the cells 10 min in advance, blocked the adhesion mediated by compounds **1** and **2** (10 μg/mL) to K562 and SK-MEL-24 cells, respectively (data not shown). This result strengthened the evidence that the cell adhesion was effectively and specifically mediated on $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins by the new beta-lactam ligands.

Wandzik et al. have reported that the protein kinase C (PKC) activator PMA (Phorbol myristate acetate) induces megakaryocytic differentiation of K562 cells upregulating $\alpha_{IIb}\beta_3$ integrin expression, which may represent a target of the assayed β-lactams [25]. We ascertained, by flow cytometry analysis, that K562 cells exposed to PMA (25 ng/mL for 40 h) express this integrin subtype, in addition to $\alpha_5\beta_1$ (Fig. 6A). Interestingly, cell adhesion of K562 cells mediated by compounds **1** and **2** was partially blocked by the selective $\alpha_{IIb}\beta_3$ antagonist tirofiban (Fig. 6B) [26]. These data seem to be indicative that both $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ integrins may contribute to mediate cell adhesion induced by compound **1** and **2** in K562 cells.

These data support the hypothesis that the novel β-lactam derivatives **1** and **2** possess an interesting activity as $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin agonists. Compound **1** displays a higher affinity toward $\alpha_5\beta_1$ integrin whereas **2** is more selective for integrin $\alpha_v\beta_3$. Both integrins are targeted by the RGD sequence [9] and it is conceivable to presume that these novel compounds could mimic this sequence. Interestingly, Aizpurua et al. [19,27] have already described substituted cyclic peptides bearing a β-lactam moiety (Fig. 1) that bind to $\alpha_v\beta_3$ integrin: the cyclic tetrapeptide with a deleted glycine residue behaves as an agonist and displays opposite angiogenic gene-regulation activity of the parent RGD peptidomimetic. Agonists have also been described for the β_2 family of integrins [28] and for $\alpha_M\beta_2$ [29]. These latter compounds are thought to stabilize the high affinity conformation of $\alpha_M\beta_2$ integrin and may function as anti-inflammatory drugs through a novel mechanism of action (perturbation of integrin de-adhesion). Recently, Vanderslice et al. [13], have reported that a small molecule agonist of $\alpha_4\beta_1$ integrin induces progenitor cell adhesion and may be an adjunct to cell-based therapy.

Until now, small molecules acting as integrin antagonists have been developed and proposed as novel drugs [8]. However, it would be advisable to develop novel small molecules that behave as integrin agonists and increase rather than decrease integrin-dependent cell adhesion. Further studies will better address the mechanism of agonism of these ligands and how they may influence cell signalling.

3. Conclusion

Three new β-lactam derivatives targeting integrins have been designed and synthesized as founders of a new family of molecules. The azetidinone is the only cyclic framework in these new compounds armed with carboxylic acid and amine terminals spaced from 9 to 14 atoms to switch on recognition by integrins. The ability of these new derivatives to modulate the adhesion of specific integrin expressing cells to immobilized fibronectin have been tested; all molecules showed a concentration-dependent enhancement in fibronectin-mediated adhesion of K562 and SK-MEL-24 cells. In particular, β-lactam **1** has a higher affinity towards $\alpha_5\beta_1$ integrin (EC_{50} of 12 nM) and β-lactam **2** is more selective for integrin $\alpha_v\beta_3$ (EC_{50} of 11 nM). The novel β-lactam derivatives

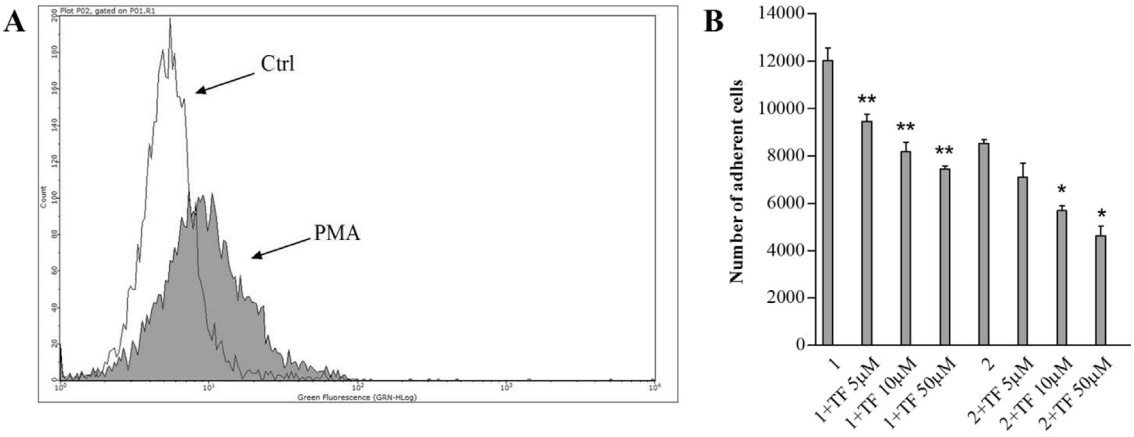


Fig. 6. Integrin $\alpha_{IIb}\beta_3$ partially mediates K562 cells adhesion to compound **1** and **2**. **A**: PMA (25 ng/mL for 40 h) induces upregulation of $\alpha_{IIb}\beta_3$ integrin expression on K562 cell surface in comparison to untreated cells (Ctrl). A representative experiment repeated three times with the same result is shown. **B**: The selective $\alpha_{IIb}\beta_3$ antagonist tirofiban (TF, 5, 10 or 50 μM) partially blocks K562 cell adhesion to wells coated with compound **1**, or **2**. Each value is the mean ± S.E.M. from four separate experiments carried out in duplicate. $^{*}p < 0.05$ compared to **2**, $^{**}p < 0.01$ compared to **1** (Newman–Keuls test after ANOVA).

described here may represent an interesting tools that increase rather than decrease $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin-dependent cell adhesion.

Work is still in progress on developing a library of compounds to assess a structure-activity analysis which could pick out the structural conditions to agonize specific classes of integrins.

4. Experimental section

4.1. General information

Commercial reagents were used as received without additional purification. ^{1}H and ^{13}C NMR spectra were recorded with an INOVA 400 or a GEMINI 200 instrument with a 5 mm probe. All chemical shifts are quoted relative to deuterated solvent signals (δ in ppm and J in Hz). Polarimetric Analyses were conducted on Unipol L 1000 "Shcmidt–Haensch" Polarimeter at 598 nm. FTIR spectra: Thermo Nicolet 380 instrument, measured as films between NaCl plates; wave numbers are reported in cm^{-1}. TLC: Merck 60 F254 plates. Column chromatography: Merck silica gel 200–300 mesh. HPLC–MS: Agilent Technologies HP1100 instrument, equipped with a ZOBRAX-Eclipse XDB-C8 Agilent Technologies column; mobile phase: H_2O/CH_3CN, 0.4 mL/min, gradient from 30 to 80% of CH_3CN in 8 min,80% of CH_3CN until 25 min, coupled with an Agilent Technologies MSD1100 single-quadrupole mass spectrometer, full scan mode from m/z = 50 to 2600, scan time 0.1 s in positive ion mode, ESI spray voltage 4500 V, nitrogen gas 35 psi, drying gas flow 11.5 mL/min, fragmentor voltage 20 V. Elemental analysis were performed on a Thermo Flash 2000 CHNS/O Analyzer.

4.2. Synthesis

Azetidinone **4** is commercial, compounds **7** [30], **12** [31], and **15** [32] were synthesized following already reported procedures.

4.2.1. tert-Butyl 2-(4-oxo-azetidin-2-yl) acetate (**5**)

In a 50 mL 3-neck flask under nitrogen, Zn powder (2.0 g, 31 mmol) and THF (10 mL) were introduced followed by TMSCl (200 μL, 1.55 mmol). After 30 min of stirring the temperature was raised to 30 ÷ 35 °C and a solution of *tert*-butylbromoacetate (1.43 mL, 15.5 mmol) in THF (20 mL) was slowly added in 30 min. After 30 min of stirring the mixture was cooled to rt and decanted, providing a limpid grey supernatant that was slowly added dropwise to a 100 mL flask under nitrogen containing a solution of **4** (500 mg, 3.88 mmol) in anhydrous THF (22 mL) at 0 °C. The mixture was stirred at rt for 3 h, quenched with ice and a saturated Seignette salt solution and extracted with AcOEt. The organic layers were dried on Na_2SO_4, filtered and concentrated in vacuum. Flash-chromatography (cyclohexane/AcOEt, 6/4) gave **5** (545 mg) as a white solid in 76% yield.

M.p. 83–84 °C; R_f 0.34 (cyclohexane/AcOEt, 1/4); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.49 (s, 9H, *t*Bu), 2.50 (dd, J = 8.8, 16.0 Hz, 1H, CHC*H*HCO$_2$*t*Bu), 2.67 (dd, J = 4.8, 16.0 Hz, 1H, CH*H*CO$_2$tBu), 2.69 (ddd, J = 1.2, 2.4, 14.8 Hz, 1H, C*H*HCHCH$_2$CO$_2$*t*Bu), 3.19 (ddd, J = 2.4, 4.8, 14.8 Hz, 1H, CH*H*CHCH$_2$CO$_2$*t*Bu), 3.95 (dddd, J = 2.4, 4.8, 4.8, 8.8 Hz, 1H, CHHC*H*CH$_2$CO$_2$tBu), 6.40 (bs, 1H, NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.0, 41.0, 43.3, 43.9, 81.4, 167.2, 170.1 ppm. IR: ν = 3238, 2964, 1763, 1735, 1398, 1261 cm^{-1}. GC–MS: R_t = 13.5 min, m/z (%) = 170 (7), 129 (13), 112 (13), 101 (10), 86 (12), 70 (36), 57 (100).

4.2.2. Benzyl 2-(4-oxo-azetidin-2-yl) acetate (**6**)

In a 50 mL 3-neck flask under nitrogen, Zn powder (2.0 g, 31 mmol) and THF (10 mL) were introduced followed by TMSCl (200 μL, 1.55 mmol). After 30 min of stirring the temperature was raised to 30÷35 °C and a solution of benzylbromoacetate (2.46 mL, 15.52 mmol) in THF (19.4 mL) was slowly added in 30 min. After 30 min of stirring the mixture was cooled to rt and decanted, providing a limpid grey supernatant that was slowly added dropwise to a 100 mL flask under nitrogen containing a solution of **4** (500 mg, 3.88 mmol) in anhydrous THF (22 mL) at 0 °C. The mixture was stirred at rt for 3 h, quenched with ice and a saturated Seignette salt (potassium sodium tartrate) solution and extracted with AcOEt. The organic layers were dried on Na_2SO_4, filtered and concentrated in vacuum. Flash-chromatography (cyclohexane/AcOEt, 1/1) gave **6** (552 mg) as a white solid in 65% yield.

M.p. 92–95 °C; R_f 0.42 (cyclohexane/tAcOEt, 1/4); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.64 (dd, J = 9.2, 16.8 Hz, 1H, C*H*HCO$_2$Bn), 2.66 (ddd, J = 1.2, 2.4, 15.2 Hz, 1H, C*H*HCHCH$_2$CO$_2$Bn), 2.78 (dd, J = 4.8, 16.8 Hz, 1H, CH*H*CO$_2$Bn), 3.15 (ddd, J = 2.4, 4.8, 15.2 Hz, 1H, CH*H*CHCH$_2$CO$_2$Bn), 3.96 (dddd, J = 2.4, 4.8, 4.8, 9.2 Hz, 1H, CHHC*H*CH$_2$CO$_2$Bn), 5.15 (s, 2H, CH$_2$Ph), 6.22 (bs, 1H, NH), 7.34–7.39 (m, 5H, Ph) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 39.8, 43.4, 43.8, 66.8, 128.3, 128.5, 128.7, 135.3, 166.9, 170.7 ppm. IR: ν = 3238, 2961, 1738, 1460, 1372, 1262 cm^{-1}. HPLC–MS: R_t = 4.82 min, m/z = 220 [M+H]$^+$, 237 [M+H$_2$O]$^+$, 439 [2M+H]$^+$.

4.2.3. tert-Butyl 4-isocyanatobenzylcarbamate (**8**)

In a 50 mL 2-neck flask with a reflux condenser under nitrogen atmosphere, a solution of (**7**) (149 mg, 0.67 mmol) and TEA (188 μL, 1.34 mmol) in anhydrous CH_2Cl_2 (7.0 mL) was cooled to 0 °C and after was added with bis(trichloromethyl) carbonate (398 mg, 1.34 mmol) in one portion. The mixture was warmed to rt and then refluxed for 3–4 h. The reaction was monitored through the increase of isocyanate IR signal (2274 cm^{-1}). The solvent was evaporated under vacuum avoiding any air exposure. The crude was extracted five times with anhydrous Et_2O under nitrogen, concentrated in vacuum, stored under nitrogen atmosphere and immediately used.

4.2.4. tert-Butyl 2-(1-(4-Boc-aminomethyl-phenylcarbamoyl)-4-oxoazetidin-2-yl) acetate (**9**)

In a 25 mL 2-neck flask under nitrogen at −78 °C, a solution of NaHMSA (Sodium bis(trimethylsilyl)amide) 1.0 M in THF (500 μL, 0.5 mmol) was added dropwise to a solution of **5** (83 mg, 0.45 mmol) in anhydrous THF (4 mL). After 15 min of stirring, a solution of isocyanate **8** (166 mg, 0.67 mmol) in THF (2 mL) was added dropwise. After completion (TLC monitoring, 30 min) the mixture was quenched with a saturated solution of NH_4Cl and extracted with AcOEt and then CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4, concentrated in vacuum and purified by flash-chromatography (CH_2Cl_2/Et_2O, 95/5) affording **9** (162 mg) as a white solid in 83% yield.

R_f 0.67 (cyclohexane/AcOEt, 2/3); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.45 (s, 9H, *t*Bu), 1.46 (s, 9H, *t*Bu), 2.68 (dd, J = 8.8, 16.0 Hz, 1H, CHC*H*HCO$_2$tBu), 2.96 (dd, J = 2.8, 16.0 Hz, 1H, CHCH*H*CO$_2$tBu), 3.24 (dd, J = 4.0, 16.4 Hz, 1H, C*H*HCHCHHCO$_2$*t*Bu), 3.35 (dd, J = 5.6, 16.4 Hz, 1H, CH*H*CHCHHCO$_2$*t*Bu), 4.28 (d, J = 5.6 Hz, 2H, CH$_2$NHCO$_2$tBu), 4.39 (m, 1H, CHHC*H*CHHCO$_2$*t*Bu), 4.80 (bs, 1H, NH), 7.25 (d, J = 8.8 Hz, 2H, arom), 7.43 (d, J = 8.8 Hz, 2H, arom), 8.47 (bs, 1H, NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.0, 28.3, 37.9, 42.6, 44.2, 47.7, 81.7 (2 × C), 119.8, 128.2, 134.9, 136.1, 140.3, 147.7, 166.9, 169.0 ppm. IR: ν = 3337, 2976, 2926, 2852, 1769, 1712, 1603, 1543, 1367, 1162 cm^{-1}. HPLC–MS: R_t = 9.71 min, m/z = 456 [M+Na]$^+$, 472 [M+K]$^+$.

4.2.5. Benzyl 2-(1-(4-Boc-aminomethyl-phenylcarbamoyl)-4-oxoazetidin-2-yl) acetate (**10**)

In a 25 mL 2-neck flask under nitrogen at −78 °C, a solution of NaHMSA (Sodium bis(trimethylsilyl)amide) 1.0 M in THF (500 μL,

0.5 mmol) was added dropwise to a solution of **6** (100 mg, 0.45 mmol) in anhydrous THF (4 mL). After 15 min of stirring, a solution of **8** (166 mg, 0.67 mmol) in THF (2 mL) was added dropwise. After completion (TLC monitoring, 30 min) the mixture was quenched with a saturated solution of NH_4Cl and extracted with AcOEt and then CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4, concentrated in vacuum and purified by flash-chromatography (CH_2Cl_2/Et_2O, 95/5) affording **10** (187 mg) in 89% yield as a colourless oil.

R_f 0.73 (cyclohexane/AcOEt, 2/3); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.47 (s, 9H, *t*Bu), 2.80 (dd, J = 8.8, 16.4 Hz, 1H, CHC*H*HCO$_2$Bn), 2.94 (dd, J = 3.2, 16.4 Hz, 1H, CHCH*H*CO$_2$Bn), 3.34 (dd, J = 2.8, 16.4 Hz, 1H, C*H*HCHCH$_2$CO$_2$Bn), 3.35 (dd, J = 4.0, 16.4 Hz, 1H, CH*H*CHCH$_2$CO$_2$Bn), 4.27 (d, J = 5.2 Hz, 2H, CH$_2$NHCO$_2$*t*Bu), 4.44 (dddd, J = 2.8, 3.2, 4.0, 8.8 Hz, 1H, CH$_2$C*H*CH$_2$CO$_2$Bn), 4.88 (bs, 1H, NHCO$_2$*t*Bu), 5.14 (d, J_{AB} = 12.4 Hz, 1H, PhCH*H*), 5.18 (d, J_{AB} = 12.4 Hz, 1H, PhC*H*H), 7.23–7.43 (m, 9H, arom), 8.42 (s, 1H, NCONH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.3, 36.8, 42.5, 44.0, 47.4, 66.7, 79.3, 119.7, 128.1, 128.2, 128.3, 128.5, 134.9, 135.2, 135.9, 147.6, 155.8, 166.5, 169.6 ppm. IR: ν = 3337, 2976, 1769, 1732, 1709, 1603, 1543, 1244, 1168 cm^{-1}. HPLC–MS: R_t = 9.84 min, m/z = 412 [M−tBu+2H]$^+$, 485 [M+H$_2$O]$^+$.

4.2.6. 4-(2-Carboxymethyl-4-oxoazetidine-1-carboxamido) phenylmethanaminium 2,2,2-trifluoroacetate (**1**)

In a 10 mL 2-neck flask under nitrogen at 0 °C, TFA (34 μL, 457 μmol) was added dropwise to a solution of **9** (33 mg, 76 μmol) in anhydrous CH_2Cl_2 (1.4 mL). The mixture was stirred at rt for 21 h, cooled at 0 °C and then treated with another aliquot of TFA (68 μL, 910 μmol). At starting material disappearing, the mixture was evaporated under vacuum yielding **1** (29 mg) in 97% yield as a light yellow waxy oil.

^{1}H NMR (400 MHz, CD_3OD): δ = 2.85 (dd, J = 8.8, 16.4 Hz, 1H, CHC*H*HCO$_2$H), 3.05 (dd, J = 3.2, 16.4 Hz, 1H, CHCH*H*CO$_2$H), 3.22 (dd, J = 3.2, 16.4 Hz, 1H, CH*H*CHCHHCO$_2$H), 3.40 (dd, J = 5.6, 16.4 Hz, 1H, C*H*HCHCHHCO$_2$H), 4.12 (s, 2H, CH$_2$N), 4.45 (m, 1H, CHHC*H*CHHCO$_2$H), 7.45 (d, J = 8.8 Hz, 2H, arom), 7.63 (d, J = 8.8 Hz, 2H, arom) ppm. ^{13}C NMR (50 MHz, D_2O): δ = 35.9, 41.4, 42.2, 47.4, 115.9 (q, J_{1CF} = 290 Hz), 121.7, 129.0, 129.4, 136.3, 149.1, 162.5 (q, J_{2CF} = 35 Hz), 168.4, 174.0 ppm. IR: ν = 3334, 2958, 2921, 1769, 1677, 1610, 1544, 1422, 1335, 1203 cm^{-1}. HPLC–MS: R_t = 1.10 min, m/z = 261 [M−TFA−NH$_3$+H]$^+$, 296 [M−TFA+H$_2$O+H]$^+$, 555 [2M−2TFA+H]$^+$, 577 [2M−TFA+Na]$^+$. Found C, 46.32; H, 4.21; N, 10.43%; $C_{15}H_{16}F_3N_3O_6$ requires C, 46.04; H, 4.12; N, 10.74%.

4.2.7. 2-(1-(4-Boc-aminomethyl-phenylcarbamoyl)-4-oxoazetidin-2-yl) acetic acid (**11**)

In a 25 mL 2-neck flask a mixture of **10** (100 mg, 0.214 mmol), anhydrous THF (2.5 mL), MeOH (2.5 mL) and Pd/C (10% w/w) (10 mg) was stirred under H_2 atmosphere (1 atm) at room temperature. At starting material consumption (2 h) the mixture was filtered and concentrated in vacuum. The desired product **11** was obtained as a light yellow solid (76 mg) in 94% yield after trituration with CH_2Cl_2.

M.p. 162–164 °C, dec.; R_f 0.69 (CH_2Cl_2/MeOH/NH_4OH, 30/10/1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.47 (s, 9H, *t*Bu), 2.81 (dd, J = 8.4, 16.8 Hz, 1H, CHC*H*HCO$_2$H), 2.98 (dd, J = 3.2, 16.8 Hz, 1H, CHCH*H*CO$_2$H), 3.33–3.42 (m, 2H, C*H*$_2$CHCH$_2$CO$_2$H), 4.28 (m, 2H, CH$_2$N), 4.46 (m, 1H, CH$_2$C*H*CH$_2$CO$_2$H), 4.83 (bs, 1H, NHCO$_2$*t*Bu), 7.25 (d, J = 8.4 Hz, 2H, arom), 7.43 (d, J = 8.8 Hz, 2H, arom), 8.46 (s, 1-H, NH) ppm. ^{13}C NMR (50 MHz, CD_3OD): δ = 29.6, 38.6, 44.2, 45.4, 46.9, 81.0, 122.0, 129.7, 137.8, 138.3, 150.7, 159.4, 169.5, 179.7 ppm. IR: ν = 3338, 2977, 2929, 1767, 1699, 1650, 1539, 1333, 1246, 1168 cm^{-1}. HPLC–MS: R_t = 4.77 min, m/z = 395 [M+H$_2$O]$^+$, 400 [M+Na]$^+$.

4.2.8. Benzyl 2-(2-(1-(4-Boc-aminomethyl-phenylcarbamoyl)-4-oxoazetidin-2-yl) acetamido) acetate (**13**)

In a 25 mL 2-neck flask, dicyclohexylcarbodiimide (DCC) (45.4 mg, 0.22 mmol) was added to a solution of **11** (51 mg, 0.135 mmol) in anhydrous CH_2Cl_2 (2.5 mL) and CH_3CN (0.5 mL) at 0 °C. Then a previously prepared solution of **12** (106.6 mg, 0.3 mmol) and stoichiometric TEA in CH_2Cl_2 (2 mL) was immediately added dropwise, followed by DMAP (4.9 mg, 0.04 mmol). The solution was warmed to rt and after complete consumption of the starting material (16 h) the mixture was quenched with H_2O (10 mL) and extract with CH_2Cl_2 (3 × 10 mL). The organic layers were dried on Na_2SO_4 and filtered. The crude was suspended in AcOEt, the residual urea by-product of DCC remained solid and was eliminated by filtration, the organic layer was concentrated in vacuum and purified by flash-chromatography (CH_2Cl_2/CH_3CN, 90/10) to afford **13** (45 mg) in 63% yield.

Light yellow waxy solid. R_f 0.75 (AcOEt), 0.79 (CH_2Cl_2/CH_3CN, 1/1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.46 (s, 9H, *t*Bu), 2.74 (dd, J = 8.4, 15.2 Hz, 1H, CHC*H*HCONH), 3.16 (m, 2H, CHC*H*HCONH, C*H*HCHCHHCONH), 3.32 (dd, J = 5.6, 16.4 Hz, 1-H, CH*H*CHCHHCONH), 4.02 (dd, J = 5.2, 18.0 Hz, 1H, NCH*H*CO$_2$), 4.10 (dd, J = 5.6, 18.4 Hz, 1H, NC*H*HCO$_2$), 4.27 (d, J = 5.6 Hz, 2H, *t*BuO-COCH$_2$N), 4.42 (m, 1-H, CHHC*H*CHHCONH), 4.85 (bs, 1H, NHCO$_2$*t*Bu), 5.16 (s, 2H, CH$_2$Ph), 6.50 (t, J = 5.2 Hz, CONH), 7.22–7.42 (m, 9H, arom), 8.51 (s, 1H, NCONH) ppm. ^{13}C NMR (50 MHz, CD_3OD): δ = 28.3, 38.1, 41.2, 42.6, 44.1, 48.3, 67.1, 79.5, 120.0, 128.1, 128.2, 128.3, 128.5, 128.6, 135.0, 135.9, 148.2, 155.9, 167.0, 169.2, 169.4 ppm. IR: ν = 3326, 2978, 2921, 1761, 1741, 1708, 1687, 1663, 1601, 1548, 1413, 1319, 1246, 1164, 1119, 1050 cm^{-1} HPLC–MS: R_t = 10.51 min, m/z = 525 [M+H]$^+$, 542 [M+H$_2$O]$^+$, 547 [M+Na]$^+$.

4.2.9. 2-(2-(1-(4-Boc-aminomethyl-phenylcarbamoyl)-4-oxoazetidin-2-yl) acetamido) acetic acid (**14**)

In a 10 mL 2-neck flask, Pd on C (10%w/w) (3 mg) was added to a solution of **13** (31 mg, 59 μmol) in anhydrous THF (350 μL) and MeOH (350 μL). The mixture was stirred under H_2 atmosphere at room temperature (1 atm) and after complete starting material consumption (2 h) it was filtered and concentrated in vacuum. The crude was then triturated with a few drops of chloroform to afford **14** after evaporation as a white solid (25 mg) in 98% yield.

R_f 0.12 (CH_3CN/MeOH, 4/1); ^{1}H NMR (400 MHz, CD_3OD): δ = 1.48 (s, 9H, *t*Bu), 2.75 (dd, J = 8.4, 14.8 Hz; 1H, CHC*H*HCONH), 3.10 (dd, J = 3.2, 16.0 Hz, 1H, C*H*HCHCHHCONH), 3.17 (dd, J = 4.4, 14.8 Hz, 1H, CHCH*H*CONH), 3.34 (dd, J = 5.6, 16.0 Hz, 1H, CH*H*CHCHHCONH), 3.91 (d, J = 17.6 Hz, 1H, NCH*H*CO$_2$), 3.97 (d, J = 17.6 Hz, 1H, NC*H*HCO$_2$), 4.21 (s, 2H, CH$_2$NHCO$_2$*t*Bu), 4.45 (m, 1H, CHHC*H*CHHCONH), 7.26 (d, J = 8.4 Hz, 2H, arom), 7.46 (d, J = 8.4 Hz, 2H, arom), 8.39 (bs, 1H, NH), 8.77 (s, 1-H, NH) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 29.6, 39.7, 42.6, 43.9, 45.4, 50.5, 81.0, 122.0, 129.7, 137.8, 138.3, 150.6, 159.4, 169.4, 173.2, 173.7 ppm. IR: ν = 3314, 2974, 2925, 1757, 1712, 1683, 1638, 1609, 1556, 1422, 1319, 1172 cm^{-1}. HPLC–MS: R_t = 2.16 min, m/z = 457 [M+Na]$^+$, 473 [M+K]$^+$.

4.2.10. 4-(2-(2-(Carboxymethylamino)-2-oxoethyl)-4-oxoazetidine-1-carboxamido) phenylmethanaminium 2,2,2-trifluoroacetate (**2**)

In a 10 mL 2-neck flask under nitrogen at 0 °C, TFA (12 μL, 165 μmol) was added dropwise to a solution of **14** (24 mg, 55 μmol) in anhydrous CH_2Cl_2 (1.4 mL). The mixture was stirred at rt for 21 h, cooled at 0 °C and then treated with another aliquot of TFA (16 μL, 220 μmol). At starting material disappearing, the mixture was evaporated under vacuum, the crude was triturated with a drop of MeOH and pentane to provide **2** as a white solid (16 mg) in 65% yield.

^{1}H NMR (400 MHz, CD_3OD): δ = 2.78 (dd, J = 8.4, 14.8 Hz, 1-H, CHC*H*HCONH), 3.13 (m, 2-H, CHCH*H*CONH + C*H*HCHCH$_2$CONH), 3.35 (m, 1H, CH*H*CHCH$_2$CONH), 3.90–3.98 (m, 2H, NHC*H*$_2$COOH), 4.11 (s, 2H, $CH_2NH_3^+$), 4.46 (m, 1H, CH_2CHCH_2CONH), 7.44 (d, J = 8.8 Hz, 2H, arom), 7.61 (d, J = 8.8 Hz, 2H, arom), 8.90 (s, 1H, NH) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 39.6, 42.6, 43.9, 44.7, 50.5, 116.8 (q, J_{1CF} = 286 Hz), 122.2, 130.8, 131.7, 140.5, 150.5, 162.3 (q), 169.4, 173.2, 173.7 ppm. IR: ν = 3387, 2949, 2921, 2839, 1772, 1707, 1678, 1654, 1621, 1560, 1458, 1417, 1020 cm^{-1}. HPLC–MS: R_t = 1.13 min, m/z = 318 [M–TFA–NH$_3$+H]$^+$, 357 [M–TFA+Na]$^+$, 373 [M–TFA+K]$^+$, 669 [2M–2TFA+H]$^+$, 691 [2M–2TFA+Na]$^+$. Found C, 45.86; H, 4.34; N, 12.38%; $C_{17}H_{19}F_3N_4O_7$ requires C, 45.54; H, 4.27; N, 12.50%.

4.2.11. (S)-Benzyl 4-oxoazetidine-2-carboxylate (**16**)

Commercially available, it has been prepared starting from dibenzylester of L-aspartic acid **15** according to the procedure reported in Ref. [28] M.p. 137–140 °C. R_f 0.6 (cyclohexane/AcOEt, 1/4); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.05–3.11 (m, 1H, CHC*H*H), 3.33 (dd, J = 6.0, 14.8 Hz, 1H, CHCH*H*), 4.22 (dd, J = 2.8, 6.0 Hz, 1H, C*H*CHH), 5.21 (s, 2H, CH_2Ph), 6.32 (bs, 1H, NH), 7.36–7.38 (m, 5H, Ph) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 43.5, 47.3, 67.4, 128.5, 128.6, 128.7, 134.8, 166.3, 170.8 ppm. HPLC–MS: R_t = 4.29 min, m/z = 206 [M+H]$^+$, 223 [M+H$_2$O]$^+$, 228 [M+Na]$^+$, 433 [2M+Na]$^+$.

4.2.12. (S)-Benzyl 1-(4-Boc-aminomethyl-phenylcarbamoyl)-4-oxoazetidine-2-carboxylate (**17**)

In a 10 mL 2-neck flask under inert atmosphere at −78 °C, NaHMSA (Sodium bis(trimethylsilyl)amide) 1.0 M in THF (190 μL, 0.190 mmol) was added dropwise to a solution of **16** (35 mg, 0.17 mmol) in anhydrous THF (1.5 mL) followed after 30 min by a solution of isocyanate **8** (64 mg, 0.26 mmol) in THF (1 mL); after starting material disappearing (30 min), the mixture was quenched with a saturated solution of NH_4Cl and extracted with AcOEt and CH_2Cl_2. The organic extracts were dried over Na_2SO_4, concentrated in vacuum and purified by flash-chromatography (CH_2Cl_2, then CH_2Cl_2/Et$_2$O 95:5) to afford **17** as a colourless viscous oil (69 mg) in 90% yield.

R_f 0.6 (cyclohexane/AcOEt, 4/1); $[\alpha]_D$ = −64.1 (c = 0.7, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.47 (s, 9-H, *t*Bu), 3.10 (dd, J = 2.8, 16.0 Hz, 1H, C*H*HCH), 3.40 (dd, J = 6.0, 16.0 Hz, 1H, CH*H*CH), 4.28 (d, J = 5.2 Hz, 2H, C*H*$_2$NH), 4.60 (dd, J = 2.8, 6.4 Hz, 1H, CHHC*H*), 4.85 (bs, 1H, NHCO$_2$*t*Bu), 5.24 (d, J_{AB} = 12.4 Hz, 1H, PhCH*H*), 5.29 (d, J_{AB} = 12.4 Hz, 1H, PhC*H*H), 7.25 (d, J = 8.4 Hz, 2H, arom), 7.35–7.38 (m, 5H, arom), 7.43 (d, J = 8.4 Hz, 2H, arom), 8.28 (s, 1H, NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.3, 41.2, 44.1, 48.9, 67.8, 79.4, 119.9, 128.2, 128.3, 128.6, 128.7, 134.7, 135.1, 135.8, 146.6, 155.8, 165.0, 168.8. IR: ν = 3424, 3342, 3060, 2974, 2921, 1777, 1744, 1708, 1683, 1601 cm^{-1}. HPLC–MS: R_t = 11.14 min, m/z = 471 [M+H$_2$O]$^+$, 476 [M+Na]$^+$.

4.2.13. (S)-1-(4-Boc-aminomethyl-phenylcarbamoyl)-4-oxoazetidine-2-carboxylic acid (**18**)

In a 10 mL 2-neck flask, Pd/C10%w/w (3 mg) was added to a solution of **17** (33 mg, 73 μmol) in anhydrous THF (400 μL) and MeOH (400 μL). The mixture was stirred under H_2 atmosphere (1 atm) at room temperature and after starting material complete consumption (2 h), it was filtered and concentrated in vacuum. The crude was then triturated with a few drops of CH_2Cl_2 and pentane to afford the desired product as a white lumpy solid (25 mg) in 95% yield.

R_f 0.03 (cyclohexane/AcOEt, 1/4); $[\alpha]_D$ = −51.5 (c = 0.4, CH_3OH); ^{1}H NMR (200 MHz, CD_3OD): δ = 1,48 (s, 9-H, *t*Bu), 3.13 (dd, J = 3.2, 16.0 Hz, 1-H C*H*HCH), 3.52 (dd, J = 6.2, 16.0 Hz, 1H, CH*H*CH), 4.21 (s, 2H, CH_2N), 4.56 (dd, J = 3.2, 6.2 Hz, 1H, CHHC*H*), 7.27 (d, J = 8.4 Hz, 2H, arom), 7.47 (d, J = 8.4 Hz, 2H, arom), 8.69 (bs, 1H, NH) ppm. ^{13}C NMR (50 MHz, CD_3OD): δ = 29.6, 42.9, 45.4, 51.1, 81.1, 122.2, 129.7, 138.0, 138.1, 149.8, 159.4, 167.9, 173.6 ppm. IR: ν = 3342, 2978, 2929, 1773, 1704, 1667, 1605, 1540, 1417, 1319, 1242, 1164 cm^{-1}. HPLC–MS: R_t = 2.03 min, m/z = 280 [M–CO$_2$*t*Bu+H$_2$O]$^-$.

4.2.14. (S)-(2-carboxy-4-oxoazetidine-1-carboxamido) phenylmethanaminium 2,2,2-trifluoroacetate (**3**)

In a 10 mL 2-neck flask under nitrogen at 0 °C a, TFA (13 μL, 165 μmol) was added dropwise to a solution of **18** (24 mg, 66 μmol) in anhydrous CH_2Cl_2 (1.2 mL). After stirring at rt for 15 h, the mixture was again cooled to 0 °C and treated with another aliquot of TFA (20 μL, 265 μmol). After starting material complete disappearing (24 h in all), the crude was evaporated under vacuum and triturated with a drop of CH_2Cl_2 and pentane to provide **3** as a white solid (22 mg) in 88% yield.

$[\alpha]_D$ = −41.8 (c = 0.25, CH_2Cl_2); ^{1}H NMR (200 MHz, CD_3OD): δ = 3.15 (dd, J = 3.0, 16.2 Hz, 1-H, C*H*HCH), 3.54 (dd, J = 6.4, 16.2 Hz, 1H, C*H*HCH), 4.11 (s, 2H, $CH_2NH_3^+$), 4.58 (dd, J = 3.0, 6.4 Hz, 1H, CHHC*H*), 7.45 (d, J = 8.4 Hz, 2H, arom), 7.63 (d, J = 8.4 Hz, 2H, arom) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ = 43.0, 44.7, 51.3, 116.8 (q), 122.4, 131.0, 131.7, 140.3, 149.7, 163.7 (q), 167.9, 173.6 ppm. IR: ν = 3411, 2954, 2921, 2851, 1777, 1691, 1625, 1552, 1462, 1323, 1204 cm^{-1}. HPLC–MS: R_t = 1.07 min, m/z = 247 [M–TFA–NH$_3$+H]$^+$, 527 [2M–2TFA+H]$^+$, 549 [2M–2TFA+Na]$^+$. Found C, 44.78; H, 3.95; N, 10.88%; $C_{14}H_{14}F_3N_3O_6$ requires C, 44.57; H, 3.74; N, 11.14%.

4.3. Pharmacology

4.3.1. Cell culture

SK-MEL-24 cells (expressing $\alpha_v\beta_3$ integrin) were from American Type Culture Collection, ATCC, Rockville, MD, USA and were routinely grown in minimum essential medium (MEM, Cambrex, Walkersville, MD, USA) supplemented with 10% foetal bovine serum (FBS; Life technologies, Carlsbad, CA, USA), non-essential aminoacids and sodium pyruvate.

K562 cells (expressing $\alpha_5\beta_1$ integrin) and Jurkat E6.1 human T cells (expressing $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrins) were also from ATCC; they were maintained as a stationary suspension culture in RPMI-1640 and glutamine with 10% FBS. Cells were kept at 37 °C under a 5% CO_2 humidified atmosphere; 40 h prior to experiments, K562 cells were treated with 25 ng/mL PMA (Phorbol 12-myristate 13-acetate, Sigma–Aldrich SRL, Milan, Italy) to induce differentiation and to increase expression of integrin cell surface [33].

4.3.2. Cell adhesion assays

The assay was performed as described in Refs. [24, 34]. Briefly, for adhesion assay on SK-MEL-24 or K562 cells 96-well plates (Corning Costar, Celbio, Milan, Italy) were coated by passive adsorption with fibronectin (10 μg/mL) overnight at 4 °C. Cells were counted with a haemocytometer and pre-incubated with various concentrations of each compound or with the vehicle (water) for 30 min at room temperature to reach ligand–receptor equilibrium. At the end of the incubation time, the cells were plated (50,000 cells per well) and incubated at room temperature for 1 h in fibronectin-coated plates. All the wells were then washed with PBS (phosphate-buffered saline) to remove non-adherent cells, and 50 μL hexosaminidase [4-nitrophenyl-N-acetyl-β-d-glucosaminide dissolved at a concentration of 7.5 mM in 0.09 M citrate buffer (pH 5) and mixed with an equal volume of 0.5% Triton X-100 in H_2O] was added. This product is a chromogenic substrate for β-N-acetylglucosaminidase, whereby it is transformed into 4-nitrophenol; absorbance was measured at 405 nm. The reaction was blocked by the addition of 100 μL of a stopping solution [50 mM glycine and

5 mM EDTA (pH 10.4)], and the plates were read in a Victor^2 Multilabel Counter (PerkinElmer, Waltham, MA, USA). Reference antagonists (Ac-Asp-Arg-Leu-Asp-Ser-OH and cyclo-Arg-Gly-Asp-D-Phe-Val) were purchased from Bachem. For adhesion assay on Jurkat E6.1 cells black 96-well plates were coated overnight at 4 °C with VCAM-1 or ICAM-1 (both 5 μg/mL). Jurkat E6.1 cells were counted and stained with CellTracker green CMFDA (12.5 μM, 30 min at 37 °C, Life Technologies); after three washes Jurkat E6.1 cells were pre-incubated with various concentrations of each compound for 30 min at 37 °C. Cells were plated (500,000 per well) on coated wells and incubated for 30 min at 37 °C. After three washes, adherent cells were lysed with 0.5% Triton X-100 in PBS (30 min at 4 °C) and fluorescence was measured (Ex485 nm/Em535 nm). Experiments were carried out in quadruplicate and repeated at least three times. Data analysis and EC_{50} values were calculated using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). The EC_{50} can be defined as the concentration of agonist required to provoke a response halfway between the baseline and maximum responses. Because the EC_{50} defines the location of the concentration–response curve for a particular compound, it is the most commonly used measure of an agonist's potency.

In another set of experiment K562 or SK-MEL-24 cells were plated (50,000 cells per well) in 96-wells plate previously coated by passive absorption with fibronectin (10 μg/mL) or directly with compound **1** or **2**, the most effective compounds under examination, dissolved in water to the final concentration of 10 μg/mL. When required, neutralizing antibodies to the β_1 (Chemicon International, Millipore) or α_v (Abcam, UK) integrin subunit (both 10 μg/mL) or a selective $\alpha_{IIb}\beta_3$ integrin antagonist tirofiban (Sigma Aldrich) [26] were added to the cells 10 min before plating cells on wells coated with compound **1** or **2**. The number of adherent cells was determined as above described.

4.3.3. Flow cytometry analysis

To determine $\alpha_{IIb}\beta_3$ integrin surface expression, K562 cells were treated with PMA 25 ng/mL for 40 h. Then cells were harvested and incubated with FITC-conjugated mouse anti-$\alpha_{IIb}\beta_3$ integrin (CD41, 10 μL for 500,000 cells) antibody (BD Biosciences) for 30 min at 4 °C. After washes, cells were resuspended in PBS and analyzed in a Guava EasyCyte (Millipore) flow cytometry; 10,000 cells/sample were analyzed.

Acknowledgements

This work was supported by MIUR (2010–2011 PRIN project: "Sintesi e applicazioni biomediche di peptidomimetici in campo oncologico") and by the University of Bologna.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.06.041.

References

[1] H. Wolfenson, I. Lavelin, B. Geiger, Dynamic regulation of the structure and functions of integrin adhesions, Dev. Cell 24 (2013) 447–458.
[2] M. Barczyk, S. Carracedo, D. Gullberg, Integrins, Cell Tissue Res. 339 (2010) 269–280.
[3] M.A. Arnaout, S.L. Goodman, J.-P. Xiong, Structure and mechanics of integrin-based cell adhesion, Curr. Opin. Cell Biol. 19 (2007) 495–507.
[4] a) N.J. Anthis, I.D. Campbell, The tail of integrin activation, Trends Biochem. Sci. 36 (2011) 191–198;
b) R. Zaidel-Bar, B. Geiger, The switchable integrin adhesome, J. Cell Sci. 123 (2010) 1385–1388;
c) M.A. Arnaout, B. Mahalingam, J.P. Xiong, Integrin structure, allostery, and bidirectional signaling, Annu. Rev. Cell Dev. Biol. 21 (2005) 381–410.
[5] J.D. Humphries, A. Byron, M.J. Humphries, Integrin ligands at a glance, J. Cell Sci. 119 (2006) 3901–3903.
[6] a) F. Ye, C. Kim, M.H. Ginsberg, Molecular mechanism of inside-out integrin regulation, J. Thromb. Haemost. 9 (Suppl. 1) (2011) 20–25;
b) B.-H. Luo, C.V. Carman, T.A. Springer, Structural basis of integrin regulation and signaling, Ann. Rev. Immunol. 25 (2007) 619–647;
c) J. Takagi, T.A. Springer, Integrin activation and structural rearrangement, Immunol. Rev. 186 (2002) 141–163.
[7] K.R. Legate, S.A. Wicksötrm, R. Fässler, Genetic and cell biological analysis of integrin outside-in signaling, Genes Dev. 23 (2009) 397–418.
[8] S.L. Goodman, M. Picard, Integrins as therapeutic targets, Trends Pharmacol. Sci. 33 (2012) 405–412.
[9] R.O. Hynes, Integrins: bidirectional, allosteric signaling machines, Cell 110 (2002) 673–687.
[10] C. Rüegg, A. Margotti, Vascular integrins: pleiotropic adhesion and signaling molecules in vascular homeostasis and angiogenesis, Cell. Mol. Life Sci. 60 (2003) 1135–1157.
[11] F. Danhier, A. Le Breton, V. Préat, RGD-based strategies to target alpha(v) beta(3) integrin in cancer therapy and diagnosis, Mol. Pharm. 9 (2012) 2961–2973 (and references cited therein).
[12] B. Cacciari, G. Spalluto, Non peptidic αvβ3 antagonists: recent developments, Curr. Med. Chem. 12 (2005) 51–70.
[13] a) P. Vanderslice, R.J. Biediger, D.G. Woodside, W.S. Brown, S. Khounlo, N.D. Warier, C.W. Gundlach, A.R. Caivano, W.G. Bornmann, D.S. Maxwell, B.W. McIntyre, J.T. Willerson, R.A.F. Dixon, Small molecule agonist of very late antigen-4 (VLA-4) integrin induces progenitor cell adhesion, J. Biol. Chem. 288 (2013) 19414–19428;
b) K. Yea, H. Zhang, J. Xie, T.M. Jones, G. Yang, B. Doo Song, R.A. Lerner, Converting stem cells to dendritic cells by agonist antibodies from unbiased morphogenic selections, PNAS 110 (2013) 14966–14971.
[14] a) M.A. Schwartz, K. McRoberts, M. Coyner, K.L. Andarawewa, H.F. Frierson Jr., J.M. Sanders, S. Swenson, F. Markland, M.R. Conaway, D. Theodorescu, Integrin agonists as adjuvants in chemotherapy for melanoma, Clin. Cancer Res. 14 (2008) 6193–6197;
b) M.H. Faridi, M.M. Altintas, C. Gomez, J.C. Duque, R.I. Vazquez-Padron, V. Gupta, Small molecule agonists of integrin CD11b/CD18 do not induce global conformational changes and are significantly better than activating antibodies in reducing vascular injury, Biochim. Biophys. Acta 1830 (2013) 3696–3710.
[15] a) C. Mas-Moruno, F. Rechenmacher, H. Kessler, Cilengitide: the first anti-angiogenic small molecule drug candidate. Design, synthesis and clinical evaluation, Anticancer Agents Med. Chem. 10 (2010) 753–768;
b) M.A. Dechantsreiter, E. Planker, B. Matha, E. Lohof, G. Hölzemann, A. Jonczyk, S.L. Goodman, H. Kessler, N-methylated cyclic RGD peptides as highly active and selective αvβ3 integrin antagonists, J. Med. Chem. 42 (1999) 3033–3040.
[16] L. Belvisi, T. Riccioni, M. Marcellini, L. Vesci, I. Chiarucci, D. Efrati, D. Potenza, C. Scolastico, L. Manzoni, K. Lombardo, M.A. Stasi, A. Orlandi, A. Ciucci, B. Nico, D. Ribatti, G. Giannini, M. Presta, P. Carminati, C. Pisano, Biological and molecular properties of a new αvβ3, αvβ5 integrin antagonist, Mol. Cancer. Ther. 4 (2005) 1670–1680.
[17] J.M. Aizpurua, J.I. Ganboa, C. Palomo, I. Loinaz, J.I. Miranda, β-Lactams RGD Cyclopeptides Containing Gamma Turns, PCT Int. Appl., WO 2006048473 A1 20060511, 2006.
[18] E. Bourguet, J.-L. Banères, J. Parello, X. Lusinchi, J.-P. Girard, J.-P. Vidal, Non-peptide RGD antagonists: a novel class of mimetics, the 5,8-disubstituted 1-azabicyclo[5.2.0]nonan-2-one lactam, Bioorg. Med. Chem. Lett. 13 (2003) 1561–1564.
[19] J.M. Aizpurua, J. Oyarbide, X. Fernandez, J.I. Miranda, J.I. Ganboa, S. Avila, J.L. Castrillo, Preparation of Pro-angiogenic β-Lactams Cyclotetrapeptides, Eur. Pat. Appl., EP 2407478 A1 20120118, 2012.
[20] N. Xi, S. Arvedson, S. Eisenberg, N. Han, M. Handley, L. Huang, Q. Huang, A. Kiselyov, Q. Liu, Y. Lu, G. Nunez, T. Osslund, D. Powers, A.S. Tasker, L. Wang, T. Xiang, S. Xu, J. Zhang, J. Zhu, R. Kendall, C. Dominguez, N-Aryl-γ-lactams as integrin αvβ3 antagonists, Bioorg. Med. Chem. Lett. 14 (2004) 2905–2909.
[21] a) F. Benfatti, G. Cardillo, S. Fabbroni, P. Galzerano, L. Gentilucci, R. Juris, A. Tolomelli, M. Baiula, A. Sparta, S. Spampinato, Synthesis and biological evaluation of non-peptide αvβ3/α5β1 integrin dual antagonists containing 5,6-dihydropyridin-2-one scaffolds, Bioorg. Med. Chem. 15 (2007) 7380–7390;
b) A. Tolomelli, M. Baiula, L. Belvisi, A. Viola, L. Gentilucci, S. Troisi, S.D. Dattoli, S. Spampinato, M. Civera, E. Juaristi, M. Escudero, Modulation of alpha(v)beta(3)- and alpha(5)beta(1)-integrin-mediated adhesion by dehydro-beta-amino acids containing peptidomimetics, Eur. J. Med. Chem. 66 (2013) 258–268.
[22] F. Schumann, A. Müller, M. Koksch, G. Müller, N. Sewald, Are beta-amino acids gamma-turn mimetics? Exploring a new design principle for bioactive cyclopeptides, J. Am. Chem. Soc. 122 (2000) 12009–12010.
[23] a) R. Cervellati, P. Galletti, E. Greco, C.E.A. Cocuzza, R. Musumeci, L. Bardini, F. Paolucci, M. Pori, R. Soldati, D. Giacomini, Monocyclic β-lactams as anti-bacterial agents: facing antioxidant activity of N-methylthio-azetidinones, Eur. J. Med. Chem. 60 (2013) 340–349;
b) P. Galletti, D. Giacomini, Monocyclic β-lactams: new structures for new biological activities, Curr. Med. Chem. 18 (2011) 4265–4283.

[24] A. Tolomelli, L. Gentilucci, E. Mosconi, A. Viola, S.D. Dattoli, M. Baiula, S. Spampinato, L. Belvisi, M. Civera, Development of isoxazoline-containing peptidomimetics as dual $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin ligands, ChemMedChem 6 (2011) 2264–2272.
[25] K. Wandzik, C. Zahn, K. Dassler, H. Fuchs, Substantial changes of cellular iron homeostasis during megakaryocytic differentiation of K562 cells, Dev. Growth Differ. 51 (2009) 555–565.
[26] a) K. Bledzka, S.S. Smyth, E.F. Plow, Integrin $\alpha_{IIb}\beta_3$: from discovery to efficacious therapeutic target, Circ. Res. 112 (2013) 1189–1200;
b) Y. Okada, J. Nishikawa, M. Semma, A. Ichikawa, Induction of integrin β_3 in PGE_2-stimulated adhesion of mastocytoma P-815 cells to the Arg-Gly-Asp-enriched fragment of fibronectin, Biochem. Pharmacol. 81 (2011) 866–872.
[27] J.M. Aizpurua, J.L. Ganboa, C. Palomo, I. Loinaz, J. Oyarbide, X. Fernandez, E. Balentovà, R.M. Fratila, A. Jiménez, J.I. Miranda, A. Laso, S. Avila, J.L. Castrillo, Cyclic RGD β-lactam peptidomimetics induce differential gene expression in human endothelial cells, ChemBioChem 12 (2011) 401–405.
[28] W. Yang, C.V. Carman, M. Kim, A. Salas, M. Shimaoka, T.A. Sprinter, A small molecule agonist of an integrin, alphaLbeta2, J. Biol. Chem. 281 (2006) 37904–37912.
[29] D. Maiguel, M.H. Faridi, C. Wei, Y. Kuwano, K.M. Balla, D. Hernandez, C.J. Barth, G. Lugo, M. Donnelly, A. Nayer, L.F. Moita, S. Schürer, D. Traver, P. Ruiz, R.I. Vazquez-Padron, K. Ley, J. Reiser, V. Gupta, Small molecule-mediated activation of the integrin CD11b/CD18 reduces inflammatory disease, Sci. Signal. 4 (2011) ra57.
[30] Y.-S. Liu, C. Zhao, E. Bergbreiter, D. Romo, Simultaneous deprotection and purification of BOC-amines based on ionic resin capture, J. Org. Chem. 63 (1998) 3471–3473.
[31] J.F. Sanz-Cervera, R. Blasco, J. Piera, M. Cynamon, I. Ibáñez, M. Murguía, S. Fustero, Improved regioselectivity in pyrazole formation through the use of fluorinated alcohols as solvents: synthesis and biological activity of fluorinated tebufenpyrad analogs, J. Org. Chem. 74 (2009) 8988–8996.
[32] A.P. Kozikowski, Y. Liao, W. Ttickmantel, S. Wang, S. Pshenichkin, A. Surin, C. Thomsen, J.T. Wroblewski, Synthesis and biology of the rigidified glutamate analogue, trans-2-carboxyazetidine-3-acetic acid (t-CAA), Bioorg. Med. Chem. Lett. 6 (1996) 2559–2564.
[33] L. Hunàkovà, J. Sedlàk, M. Klobusická, B. Chorváth, Phorbol ester (TPA)-induced differential modulation of cell surface antigens in human pluripotential leukemia (K-562) cell line: effects of protein kinase inhibitors with broad- and PKC selective inhibitory activity, Neoplasma 42 (1995) 249–253.
[34] a) A.R. Qasem, C. Bucolo, M. Baiula, A. Spartà, P. Govoni, A. Bedini, D. Fascì, S. Spampinato, Contribution of alpha4beta1 integrin to the antiallergic effect of levocabastine, Biochem. Pharmacol. (2008) 751–762;
b) S.D. Dattoli, R. De Marco, M. Baiula, S. Spampinato, A. Greco, A. Tolomelli, L. Gentilucci, Synthesis and assay of retro-α4β1 integrin-targeting motifs, Eur. J. Med. Chem. 73 (2014) 225–232.