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DOI: 10.1002/cmdc.201100372

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Development of Isoxazoline-Containing Peptidomimetics as Dual $\alpha_v\beta_3$ and $\alpha_5\beta_1$ Integrin Ligands

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Isoxazoline-containing peptidomimetics, designed to be effective $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin ligands, were synthesized through an original procedure involving *N*,*O*-bis(trimethylsilyl)hydroxyamine conjugate addition to alkylidene acetoacetates, followed by intramolecular hemiketalization. To mimic the RGD recognition sequence, basic and acidic terminal appendages were introduced, and the final products were tested in cell adhesion inhibition assays. All the synthesized compounds proved to be excellent ligands for both integrin receptors, and a strong influence on intracellular signaling and phosphorylation pathways was demonstrated by evaluation of fibronectin-induced phosphorylation of ERK. The molecular basis of the observed inhibitory activity was suggested on the results of docking experiments.

Introduction

Development of novel tools for the early detection,[1] diagnosis,[2] and therapy[3] of cancer is one of the most important goals in medicinal chemistry. The complex mechanism of cell–cell and cell–matrix adhesion is crucial for developmental processes, as such connections lead to cellular communication and signaling. Among the broad spectrum of cell-adhesion molecules, integrin receptors,[4] a large family of heterodimeric transmembrane glycoproteins, play a fundamental role, and thus they are involved in the pathogenesis of several diseases such as atherosclerosis, osteoporosis, cancer, and a variety of inflammatory disorders. The $\alpha_v\beta_3$ integrin is present at low levels on healthy tissues, whereas it is overexpressed in certain pathologies such as metastatic melanoma, late-stage glioblastoma, and breast and prostate tumors.[5] It is also preferentially expressed on cancer blood vessels, mediating angiogenesis initiated by basic fibroblast growth factor (bFGF) or tumor necrosis factor-α (TNF-α).[6] Another member of this class of receptors, $\alpha_5\beta_1$ integrin, was first identified as an attachment point for cell penetration by certain viruses and bacteria,[7] but it has also recently been reported to play a facilitating role in cancer cell invasion during migration through connective tissues, through the generation of enhanced contractile forces.[8] The $\alpha_5\beta_1$ integrin has also been unambiguously identified as a pro-angiogenic receptor, as antagonists to its extracellular matrix partner fibronectin are able to block growth-factor- and tumor-induced angiogenesis.[9] Simultaneous blockade of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins was reported to inhibit bFGF-induced angiogenesis,[10] and evidence of cross-talk between the two receptors has been provided.[11] Therefore, the identification of synthetic ligands that are able to engage with these cell-surface receptors could aid in the design of imaging biomarkers for early detection of cancer and assessment of therapy response,[12] as well as in the engineering of cell-targeted anticancer agents.[13]

Processes such as angiogenesis, which involve several mechanisms, can be addressed by targeting multiple pathogenic pathways with a single compound.[14] This approach may provide enhanced efficacy because pharmacokinetic problems related to the administration of more than one drug can be minimized. Antagonists to both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins may have a synergistic effect and could suppress tumor angiogenesis. Some selected examples of dual $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin inhibitors[15] are shown in Figure 1.

The design and synthesis of dual non-peptidic integrin inhibitors has generally been planned on the basis of the Arg-Gly-Asp (RGD) motif, which is present in a wide range of extracellular matrix (ECM) proteins.[16] X-ray crystallographic analysis of the complex between $\alpha_v\beta_3$ integrin and the privileged synthetic ligand cilengitide[17] shows that the recognition sequence binds to the receptor mainly through electrostatic interactions with regions in the protein having opposite charges: arginine interacts with two aspartic acid residues situated in the α unit of the protein, while the carboxylate group of the ligand binds to the metal-ion-dependent adhesion site (MIDAS). No high-resolution structural information regarding $\alpha_5\beta_1$ integrin is currently available to understand the molecular

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201100372.

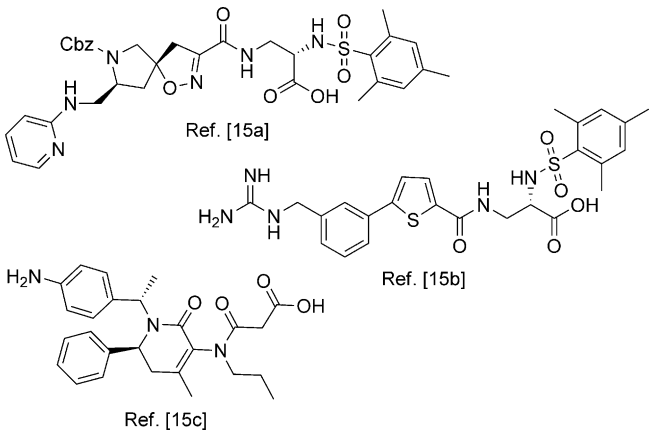


Figure 1. Selected examples of dual $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin inhibitors reported in the literature.

basis of ligand–receptor binding, but homology models have been proposed on the basis of the $\alpha_v\beta_3$ integrin–cilengitide complex structure[18] and through binding pocket mapping using a gain-of-function approach.[19]

Several research groups have been involved in the development of small constrained peptidomimetics of the RGD motif, the enhanced bioavailability of which could result in more promising drugs.[20] Typically, a rigid heterocyclic scaffold is incorporated as the central core of a small non-peptidic molecule, having acidic and basic moieties properly oriented and distanced for binding. Over the last few years, extensive studies have been undertaken regarding the synthesis of sequences that contain unusual linear or cyclic amino acids.[21] Thus, the design and synthesis of new enantiopure non-proteinogenic amino acids represent a central issue for chemists working in this broad research area. In a similar way, the preparation of small heterocyclic rings through simple and efficient routes is receiving increased attention for their potential application as scaffolds in the development of bioactive compound libraries.[22]

In this context, isoxazolidines have been extensively explored both as synthetic intermediates[23] and as conformational constraints.[24] These five-membered heterocycles may be envisaged as masked amino acids, as 1,3-amino alcohol equivalents, or as furanose ring mimics in the preparation of bioactive compounds. Indeed, isoxazolines have been incorporated as conformational constraint elements in several transcriptional activators.[25] In a similar way, analogues of the antimitotic combretastatin containing an isoxazoline or isoxazole ring have been reported,[26] and the synthesis of nucleoside analogues containing an isoxazolidinic and isoxazolinic ring is of current significant interest in the development of antiviral and anticancer agents.[27]

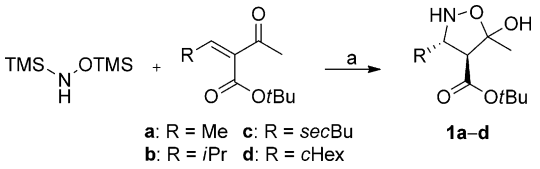
We recently described the synthesis of ethyl 5-hydroxyisoxazolidine-4-carboxylate and related functionalized isoxazolines through a Lewis-acid-induced Michael addition of hydroxyamine derivatives to alkylideneacetoacetates, followed by intramolecular hemiketal formation.[28] On the basis of our experience in the introduction of heterocyclic scaffolds as central cores of RGD peptidomimetics,[15c] we decided to test these five-membered rings as scaffolds with the goal of obtaining a small library of functionalized isoxazoline-based integrin ligands.

Results and Discussion

Chemistry

The conjugate addition of nitrogen-containing nucleophiles to electron-deficient olefins is one of the most frequently used and versatile methods for C–N bond construction in organic chemistry.[29] Alkylidene malonates have been extensively employed as Michael acceptors.[30] In particular, a chiral Lewis-acid-catalyzed Michael addition of hydroxyamino derivatives to alkylidene malonates has been reported by our research group.[31] The use of acetoacetates in place of malonates in this field is rather unusual and has the advantage of introducing a reactive keto functionality that may be further elaborated.[28a]

According to these considerations, the isoxazolidine **1** was prepared by treatment of *tert*-butylalkylidene acetoacetates with *N*,*O*-bis(trimethylsilyl)hydroxyamine in the presence of a catalytic amount of zinc trifluoromethanesulfonate (Scheme 1). Reaction conditions were optimized on the basis of our previous work in order to avoid the formation of an oxime byproduct resulting from the undesired 1,2-addition process.[32] Direct addition of silica gel in the reaction workup induced rapid conversion of the intermediate adduct to *trans*-5-hydroxyisoxazolidine-4-carboxylate **1a–d** via intramolecular hemiketalization. The procedure, previously reported for ethyl and methyl esters, was successfully applied to *tert*-butylalkylidene acetoacetates.[32]

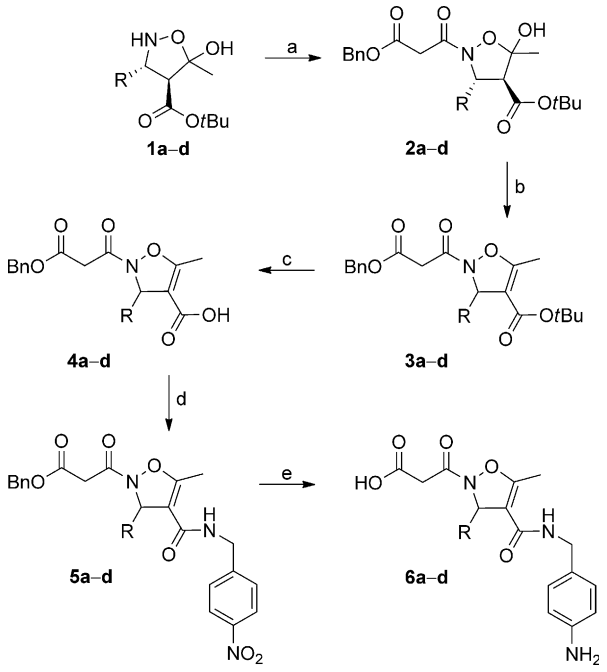


Scheme 1. Synthesis of 5-hydroxyisoxazolidine-4-carboxylates **1a–d** via conjugate addition of *N*,*O*-bis(trimethylsilyl)hydroxyamine to alkylidene acetoacetates: a) $Zn(OTf)_2$ (5%), CH_2Cl_2.

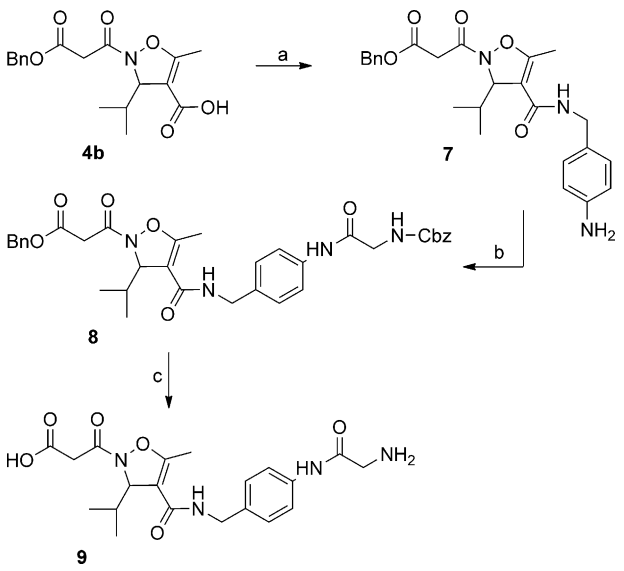
Introduction of the heterocyclic core into a peptidomimetic structure that possesses the fundamental features for receptor affinity required selection of the proper appendages to mimic aspartate and arginine side chains. The basicity and length of the guanidine-mimicking group was found to play a central role, while the presence of a carboxylate function to mimic aspartate is necessary to create an electrostatic interaction with the metal cation in the binding pocket. We recently synthesized a small library of RGD mimetics containing 5,6-dihydropyridin-2-ones and evaluated the effect of various side chains on the inhibition of adhesion.[15c] This study suggested that linking 4-aminobenzylamine and malonic acid to a rigid scaffold, as basic and acidic termini, respectively, may provide access to efficient $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin ligands.

Therefore, racemic *trans*-5-hydroxyisoxazolidine-4-carboxylates **1a–d** were acylated at the nitrogen atom by treatment with benzyl malonyl chloride, generated in situ from the corresponding acid and thionyl chloride, in the presence of pyridine (Scheme 2). The reaction afforded compounds **2a–d** in yields ranging from 60 to 65%. Dehydration of the isoxazolidines **2a–d** to the corresponding unsaturated racemic isoxazolines **3a–d** was accomplished by transformation of the hydroxy moiety into a mesylate group; subsequent base-induced elimination led to loss of the stereocenter at position 4 of the heterocycle (85–90% yield). The *tert*-butyl and benzyl esters were chosen as terminal groups to effect their selective removal. Indeed, treatment of **3a–d** with excess trifluoroacetic acid (TFA) in dichloromethane afforded acids **4a–d** in nearly quantitative yield. Coupling with 4-nitrobenzylamine by following the protocol usually applied for peptide synthesis (EDCI/HOBt/Et_3N) allowed introduction of the arginine mimetic chain, bearing a terminal nitro group as a masked form of the aromatic amino moiety. Compounds **5a–d** were isolated after purification by flash chromatography on silica gel in yields ranging from 60 to 70%. Finally, hydrogenation of **5a–d** with palladium/carbon as catalyst at atmospheric pressure led to simultaneous removal of the benzyl ester moiety and reduction of the nitro group to amine, affording compounds **6a–d** in excellent yields (70–85%). Purification of the final isoxazoline compounds with Dowex 50WX2-200 ion-exchange resin was always performed before evaluation of bioactivity.

With the aim of determining whether elongated molecules are better suited for fitting into the receptor pocket and if a more basic aliphatic amine could create stronger interactions with the aspartate groups present in the integrin binding site, we designed the synthesis of **9** starting from **4b** (Scheme 3). By direct reaction of **4b** with 4-aminobenzylamine in the presence of HBTU and triethylamine, regioisomer **7** was exclusively obtained in 80% yield, thus confirming that the aromatic amine is less reactive than the aliphatic amine. Elongation was carried out by coupling **7** with *N*-Cbz-glycine chloride, generated in situ from the corresponding acid and thionyl chloride, in the presence of triethylamine to obtain **8** in 58% yield. Finally, simultaneous removal of the benzyl ester and the benzyloxycarbonyl protecting group by hydrogenation on Pd/C followed by treatment with Dowex 50WX2-200 ion-exchange resin afforded **9** in nearly quantitative yield.



Scheme 2. Synthesis of RGD mimetics **6a–d**: a) monobenzyl malonate, $SOCl_2$, pyridine, CH_2Cl_2, RT, 60–65%; b) CH_3SO_2Cl, Et_3N, CH_2Cl_2, RT, 85–90%; c) TFA, RT, >95%; d) 4-nitrobenzylamine, EDCI, HOBt, Et_3N, CH_2Cl_2/DMF, RT, 60–70%; e) H_2/Pd/C, MeOH, RT, 70–85%.



Scheme 3. Synthesis of RGD mimetic **9b**: a) 4-aminobenzylamine, HBTU, Et_3N, CH_2Cl_2, RT, 80%; b) *N*-Cbz-Gly, $SOCl_2$, Et_3N, CH_2Cl_2, RT, 58%; c) H_2/Pd/C, MeOH, RT, >95%.

Pharmacology

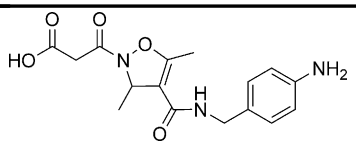
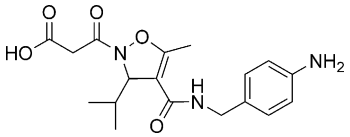
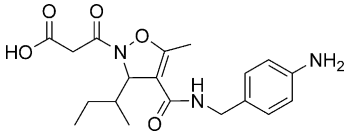
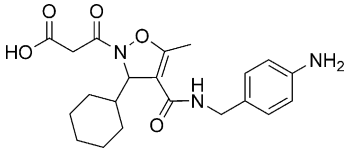
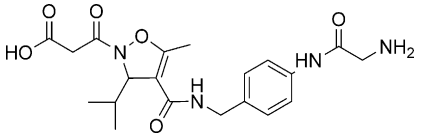
Inhibition of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin-mediated cell adhesion

The ability of racemic compounds **6a–d** and **9** to inhibit the adhesion of K562 (human erythroleukemia expressing $\alpha_5\beta_1$ integrin) or SK-MEL-24 (human malignant melanoma expressing $\alpha_v\beta_3$ integrin) cells to immobilized fibronectin was evaluated. These cell models are widely used to investigate potential antagonists of $\alpha_v\beta_3$ integrin-mediated SK-MEL-24 cell adhesion.[33] The bioassays were not performed on intermediates **1a–d**, **2a–d**, **3a–d**, **4a–d** and **5a–d**, which do not possess the functional groups and spacing required for receptor affinity. In these experiments, cells were seeded onto plates coated with different substrata and allowed to adhere before quantitation of the number of adherent cells in the presence of increasing concentrations of test compounds. Under these conditions, no significant cell adhesion was observed for bovine serum albumin (BSA)-coated plates (negative control) or nonspecific substrate-coated plates (i.e., collagen I for SK-MEL-24 expressing

$\alpha_v\beta_3$ integrin and poly-L-lysine for K562 expressing $\alpha_5\beta_1$ integrin; data not shown). The results are summarized in Table 1.

The ability of the new compounds to inhibit the adhesion of SK-MEL-24 and K562 cells to fibronectin or vitronectin was compared with that of the standard compounds, Ac-Asp-Arg-Leu-Asp-Ser-OH and H-Gly-Arg-Gly-Asp-Asn-Pro-OH, known to be potent inhibitors of cell adhesion mediated by $\alpha_v\beta_3$ integrin.[34] Among the five new RGD mimetics tested, compound **6b** exhibited the highest potency as an inhibitor of cell adhesion mediated by $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. The IC_{50} values against both integrin-mediated cell adhesion events were in the nanomolar range (Entry 2, Table 1). The results listed in Table 1 show that the isoxazoline ring is an effective scaffold for inducing the proper orientation of the Asp- and Arg-mimicking chain, as smaller members of the library displayed excellent inhibitory activities toward adhesion mediated by both receptors, and can thus be regarded as dual inhibitors.

Table 1. Inhibition of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin-mediated cell adhesion to fibronectin (FN) in the presence of compounds **6a–d**, **9**, or reference standards.

Entry	Compd	IC_{50}[a] [nM] $\alpha_v\beta_3$	$\alpha_5\beta_1$
1	**6a** 	32 ± 3	12 ± 4
2	**6b** 	8.8 ± 0.6	1.05 ± 0.3
3	**6c** 	360 ± 70	1320 ± 80
4	**6d** 	20 ± 6	1030 ± 50
5	**9** 	15 500 ± 900	> 100 000
6[b,c]	Ac-Asp-Arg-Leu-Asp-Ser-OH	25 ± 3	> 100 000
7[b]	H-Gly-Arg-Gly-Asp-Asn-Pro-OH	234 ± 32	> 100 000

[a] Values represent the mean ± SD of three experiments. [b] Reference compounds. [c] See reference [15c].

Compound **6a**, which has a smaller methyl substituent on the isoxazoline ring, maintained notable efficacy, showing IC_{50} values only tenfold less potent than compound **6b** (IC_{50}: 32 nM versus $\alpha_v\beta_3$ integrin and 12 nM versus $\alpha_5\beta_1$ integrin, Entry 1). The *sec*-butyl-substituted derivative **6c** showed decreased inhibitory activity (Entry 3), but an increase in selectivity toward $\alpha_v\beta_3$ integrin. A strong preference of compound **6d** for $\alpha_v\beta_3$ integrin was observed, as it has an IC_{50} value of 20 nM as an $\alpha_v\beta_3$ integrin antagonist, whereas its IC_{50} value toward $\alpha_5\beta_1$ is 1 μM (Entry 4). Finally, compound **9**, which was designed to verify the effect of elongation and a terminal aliphatic amine on adhesion inhibition, exhibited poor IC_{50} values, in the millimolar to sub-millimolar range (Entry 5).

In agreement with previous studies, Ac-Asp-Arg-Leu-Asp-Ser-OH displayed nanomolar inhibitory activity toward the cell-adhesive capacity driven selectively by $\alpha_v\beta_3$ integrin, whereas the peptide H-Gly-Arg-Gly-Asp-Asn-Pro-OH had IC_{50} values in the sub-micromolar range against $\alpha_v\beta_3$ integrin. Interestingly, compounds **6a** and **6b** are more potent than the reference compounds Ac-Asp-Arg-Leu-Asp-Ser-OH and H-Gly-Arg-Gly-Asp-Asn-Pro-OH, as they inhibit cell adhesion mediated by $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin with IC_{50} values lower than those of the reference compounds. Moreover, as compound **6b** exhibited the highest potency as an inhibitor of cell adhesion to fibronectin, we tested its ability to block the adhesion of SK-MEL-24 cells to vitronectin relative to the standard peptide Ac-Asp-Arg-Leu-Asp-Ser-OH. Compound **6b** showed an IC_{50} value of 3.14 nM, while the reference peptide showed an IC_{50} value in the sub-micromolar range (IC_{50}: 0.33 μM) versus vitronectin-mediated cell adhesion.

Effect of an integrin antagonist on fibronectin-induced ERK phosphorylation

The mechanism by which components of the ECM generate intracellular signaling through integrins requires increased phosphorylation of cytoplasmic second messengers. Phosphorylation of ERK plays a central role in fibronectin-mediated survival signaling through integrins: in fact, cell adhesion activates ERK by binding of $\alpha_5\beta_1$ integrins at the cell surface to ECM proteins such as fibronectin.[35] Therefore, we investigated the most effective compound **6b** on fibronectin-induced phosphorylation of ERK in K562 cells, which express $\alpha_5\beta_1$ integrin.

K562 cells were serum-starved in RPMI-1640 containing 1% fetal bovine serum (FBS) for 16 h; thereafter, they were pre-incubated with compound **6b** for 30 min in suspension and plated for 30 min on fibronectin or poly-L-lysine (used as non-specific substrate). In agreement with other studies,[35] the ERK

pathway is activated in K562 cells exposed to phorbol-12-myristate-13-acetate (PMA, 65 nM; Figure 2). In cells exposed to fibronectin, a significant increase in phosphorylated ERK is observed after 60 min, whereas no change is detected for shorter exposure times, as previously described. This late ERK phosphorylation induced by fibronectin has been previously reported.[35]

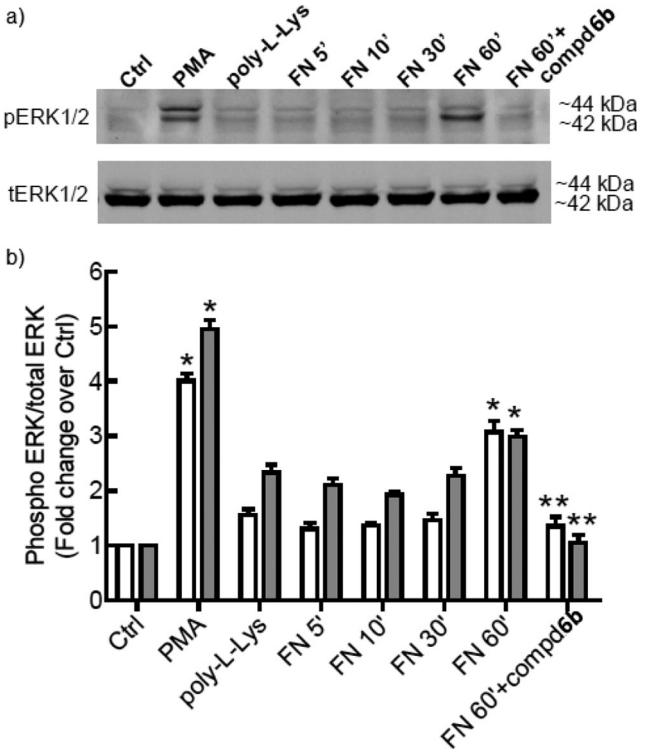


Figure 2. Compound **6b** prevents fibronectin-induced phosphorylation of ERK. K562 cells were serum-starved in RPMI-1640 containing 1% FBS for 16 h; cells were then pre-incubated with compound **6b** for 30 min in suspension. The cells were kept in suspension (Ctrl) or plated on fibronectin (FN) or on poly-L-lysine (poly-L-Lys). After 30 min, cells were lysed, and lysates were analyzed by Western blot using an antibody directed against phosphorylated ERK (pERK) or total ERK (tERK). a) Western blot shows that control cells exposed to PMA (65 nM) or plated on FN had a much stronger signal for phosphorylated ERK than those plated on poly-L-lysine (used as nonspecific substrate). Pre-incubation with compound **6b** before plating on fibronectin caused a significant decrease in the amount of phosphorylated ERK in K562 cells. b) Densitometric analysis of the bands (mean ± SEM; $n = 6$): p42 (□), p44 (■); the amount of phosphorylated ERK is normalized to the total amount of ERK. Densitometric quantification confirmed that compound **6b** specifically decreases fibronectin-induced phosphorylation of ERK. In control cells, as expected, PMA induced a significant increase in ERK phosphorylation; $^{*}p < 0.01$ versus Ctrl, $^{**}p < 0.001$ versus FN60′ (Newman–Keuls test after ANOVA).

Western blots showed that for K562 cells not pre-incubated with compound **6b**, exposure to fibronectin for 60 min elicited a much stronger signal for phosphorylated ERK than those plated on poly-L-lysine (Figure 2a). Pre-incubation with compound **6b** (1 μM) caused a significant decrease in the amount of fibronectin-induced ERK phosphorylation in K562 cells.

Molecular docking

To interpret the inhibitory activity observed for compound **6b** in the $\alpha_v\beta_3$-mediated cell adhesion assays at the molecular level, a computational model for the binding of **6b** to $\alpha_v\beta_3$ integrin was built by using a previously established and validated docking approach.[20c] Starting from the X-ray crystal structure of the extracellular segment of integrin $\alpha_v\beta_3$ in complex with the cyclic pentapeptide ligand cilengitide (PDB ID: 1L5G),[17] automated docking calculations were carried out with the Glide software package[36] according to the procedure described in the Experimental Section below. The docking protocol was successful in reproducing the crystallographic binding mode of cilengitide at the interface of the α and β subunits, forming all the specific electrostatic and hydrogen bond interactions. The docking protocol was then applied to small libraries of cyclic and linear RGD peptidomimetics to evaluate their ability to properly fit into the receptor site. Good agreement between docking results and experimental biological data was observed.[20c,37] In all the calculations, the experimentally observed binding mode of cilengitide with $\alpha_v\beta_3$ integrin was taken as a reference model for the analysis of docking results in terms of ligand–protein interactions.

In this work, the Glide docking protocol was applied to compound **6b** (both the *R* and *S* enantiomers) to generate a structural model for the interaction with the ligand binding site of the $\alpha_v\beta_3$ integrin receptor. Automated docking calculations of both enantiomers produced top-ranked poses conserving the key ionic interactions. As shown in Figure 3, the acidic and basic pharmacophore groups of compound (*R*)-**6b** act like an electrostatic clamp, interacting with charged regions of the receptor binding site. In particular, the ligand carboxylate group is coordinated to the metal cation in the MIDAS region of the β_3 subunit, and the ligand aromatic amine moiety interacts with the negatively charged side chains of Asp218 and Asp150 in the α_v subunit. Further stabilizing interactions involve the formation of hydrogen bonds between the ligand carboxylate group and the backbone amide hydrogen atoms

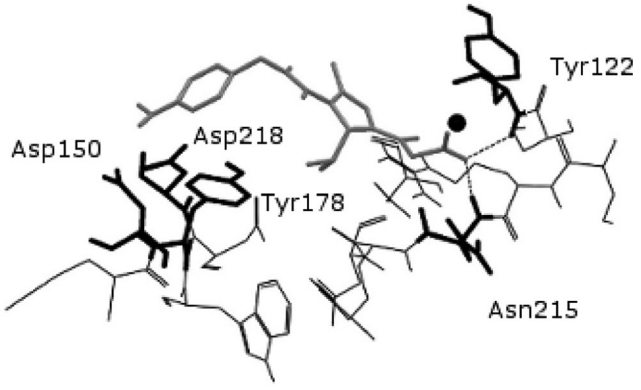


Figure 3. Docking best pose of compound (*R*)-**6b** (thick grey lines) into the crystal structure of the extracellular domain of $\alpha_v\beta_3$ integrin. Selected integrin residues involved in interactions with the ligand are shown in black. The Mn^{2+} ion at MIDAS is shown as a dark CPK sphere. Nonpolar hydrogen atoms have been removed for clarity.

of Asn 215 and Tyr 122 in the β unit. A ring-stacking effect between the ligand 4-aminobenzylamine and the Tyr 178 side chain of the α_v subunit is also observed.

Conclusions

A novel class of isoxazoline-containing peptidomimetics designed as effective $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin ligands was developed on the basis of the well-known interactions of these transmembrane receptors with bioactive ligands. For this purpose, the isoxazolidine ring was synthesized by following an original procedure involving *N,O*-bis(trimethylsilyl)hydroxyamine conjugate addition to alkylidene acetoacetates, followed by intramolecular hemiketalization. Appendages that mimic arginine and aspartate side chains were introduced, and the final products were tested in cell adhesion inhibition assays. Most of the synthesized compounds proved to be excellent integrin ligands, showing IC_{50} values in the nanomolar range for both receptors. The effect of the most active compound **6b** on fibronectin-induced phosphorylation of ERK also allowed verification that ligand–receptor binding has a strong influence on intracellular signaling and phosphorylation pathways. Moreover, docking experiments yielded insight into the possible interactions between **6b** and $\alpha_v\beta_3$ integrin, thus providing information on the molecular basis of the observed inhibitory activity. Because $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins play such an important role in tumor angiogenesis, the new compounds reported herein will likely serve as crucial leads for the development of therapeutic and diagnostic tools.

Experimental Section

Chemistry

General: All chemicals were purchased from commercial suppliers and were used without further purification. Anhydrous solvents were purchased in sure-seal bottles over molecular sieves and were used without further drying. Flash chromatography was performed on silica gel (230–400 mesh). Dowex 50WX2-200(H) ion-exchange resin was used for purification of free amino acids. NMR Spectra were recorded with Varian Gemini 200, Mercury Plus 400, or Unity Inova 600 MHz spectrometers. Chemical shifts (δ) are reported in ppm relative to the solvent peak of $CDCl_3$ set at $\delta=$ 7.27 ppm (^{1}H NMR) or $\delta=$77.0 ppm (^{13}C NMR), CD_3OD set at $\delta=$ 3.31 ppm (^{1}H NMR) or $\delta=$49.0 ppm (^{13}C NMR), or D_2O set at $\delta=$ 4.79 ppm (^{1}H NMR). Coupling constants (*J*) are given in Hz. LC–MS analyses were performed on an HP1100 liquid chromatograph coupled with an electrospray ionization mass spectrometer (LC–ESIMS), using H_2O/CH_3CN as solvent at 25 °C (positive scan 100–500 *m/z*, fragmentor 70 V). Compounds **1a–d** were prepared by following reported procedures.[28b] Complete characterization of intermediates bearing the isopropyl side chain and of final compounds are reported below. Data for all other intermediates are reported in the Supporting Information.

General procedure for the synthesis of *N*-malonamido derivatives 2a–d: $SOCl_2$ (1.5 mmol, 1.5 equiv) was added dropwise to a stirred solution of benzyl malonic acid (1.5 mmol, 1.5 equiv) in dry CH_2Cl_2 (5 mL) at 0 °C. After removing the ice bath, the solution was stirred for 30 min at room temperature, and then pyridine (5 mmol, 5 equiv) and isoxazolidine **1a–d** (1 mmol) were added. The solution was left to stir overnight, and then the reaction mixture was diluted with CH_2Cl_2 (10 mL) and washed with H_2O (2× 10 mL). The organic layer was dried (Na_2SO_4) and filtered, and the solvent was removed in vacuo. Purification of the crude residue by flash chromatography on silica gel, (eluent cyclohexane/EtOAc 9:1) afforded compounds **2a–d** as racemic mixtures of *trans* isomers.

***tert*-Butyl 2-[3-(benzyloxy)-3-oxopropanoyl]-5-hydroxy-3-isopropyl-5-methylisoxazolidine-4-carboxylate (2b)**: Yellow oil (292 mg, 66%): ^{1}H NMR (200 MHz, $CDCl_3$): $\delta=$0.71 (d, $J=$7.0 Hz, 3H), 0.88 (d, $J=$7.0 Hz, 3H), 1.40 (s, 9H), 1.54 (s, 3H), 1.88 (m, 1H), 2.87 (d, $J=$6.6 Hz, 1H), 3.43 (d, $J=$15.6 Hz, 1H), 3.60 (d, $J=$15.6 Hz, 1H), 4.51 (m, 1H), 4.61 (bs, 1H), 5.08 (s, 2H),*7*.29 ppm (s, 5H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta=$17.7, 18.6, 23.0, 27.9, 32.2, 41.2, 58.8, 65.0, 67.1, 82.8, 105.7, 128.2, 128.5, 128.6, 135.4, 168.0, 168.7, 169.2 ppm; LC–MS (ESI): $t_R=$10.4 min, *m/z*: 444 [*M*+Na], 865 [2*M*+Na].

General procedure for the synthesis of dehydration of 2a–d to 3a–d: CH_3SO_2Cl (116 μL, 1.5 mmol, 1.5 equiv) and Et_3N (348 μL, 2.5 mmol, 2.5 equiv) were added in a single portion to a stirred solution of **2a–d** (1 mmol) in dry CH_2Cl_2 (5 mL) at room temperature. The solution was stirred at room temperature and stopped upon disappearance of the starting material spot (by TLC), typically after 1 h. The reaction mixture was then diluted with CH_2Cl_2 (10 mL) and washed with H_2O (2×10 mL). The organic layer was dried (Na_2SO_4) and filtered, and the solvent was removed in vacuo. Purification of the crude residue by flash chromatography on silica gel (eluent cyclohexane/EtOAc 9:1) afforded compounds **3a–d**.

***tert*-Butyl 2-[3-(benzyloxy)-3-oxopropanoyl]-3-isopropyl-5-methyl-2,3-dihydroisoxazole-4-carboxylate (3b)**: Yellow oil (359 mg, 89%): ^{1}H NMR (200 MHz, $CDCl_3$): $\delta=$0.71 (d, $J=$6.6 Hz, 3H), 0.89 (d, $J=$6.6 Hz, 3H), 1.41 (s, 9H), 1.55 (m, 1H), 2.01 (s, 3H), 3.44 (d, $J=$15.8 Hz, 1H), 3.60 (d, $J=$15.8 Hz, 1H), 5.08 (s, 2H), 5.12 (bs, 1H), *7*.36 ppm (m, 5H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta=$10.9, 15.6, 19.7, 29.0, 31.3, 40.9, 66.9, 68.1, 80.7, 105.3, 128.1, 128.2, 128.4, 135.0, 161.1, 162.2, 166.3, 169.4 ppm; LC–MS (ESI): $t_R=$ 12.4 min, *m/z*: 404 [*M*+1], 829 [2*M*+Na].

General procedure for *tert*-butyl ester hydrolysis (compounds 4a–d): TFA (15 equiv, 15 mmol, 1.15 mL) was added to a stirred solution of **3a–d** (1 mmol) in CH_2Cl_2 (2 mL) at 0 °C. After 2 h, the mixture was washed with H_2O (2×5 mL), the organic layer was dried (Na_2SO_4), filtered, and the solvent was removed in vacuo. The acids **4a–d** were used in the following step without further purification.

2-[3-(Benzyloxy)-3-oxopropanoyl]-3-isopropyl-5-methyl-2,3-dihydroisoxazole-4-carboxylic acid (4b): Yellow oil (340 mg, 98%): ^{1}H NMR (200 MHz, $CDCl_3$): $\delta=$0.80 (d, $J=$6.8 Hz, 3H), 0.98 (d, $J=$ 6.8 Hz, 3H), 2.12 (s, 3H), 2.20 (m, 1H), 3.53 (d, $J=$16.0 Hz, 1H), 3.74 (d, $J=$16.0 Hz, 1H), 5.16 (s, 2H), 5.26 (bs, 1H), *7*.34 ppm (m, 5H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta=$11.4, 15.4, 19.8, 23.9, 31.2, 40.9, 67.3, 103.7, 128.3, 128.4, 128.5, 134.9, 164.8, 166.4, 168.6, 169.9 ppm; LC–MS (ESI): $t_R=$9.2 min, *m/z*: 348 [*M*+1], 370 [*M*+Na], 717 [2*M*+ Na].

General procedure for coupling of 4a–d with *p*-nitrobenzylamine (compounds 5a–d): EDCI (1.1 equiv, 0.55 mmol, 105 mg) and Et_3N (2.1 equiv, 1.05 mmol, 146 μL) were added to a stirred solution of acid **4** (0.5 mmol) in dry CH_2Cl_2 (2 mL) under N_2 atmosphere. After 30 min, HOBt (1.1 equiv, 0.55 mmol, 74 mg) and *p*-nitrobenzylamine·HCl (1.1 equiv, 0.55 mmol, 103 mg) were added. The solution was stirred overnight and then the mixture was diluted with CH_2Cl_2 (8 mL) and washed with acidic H_2O (2×5 mL, pH 3) and basic H_2O (2×5 mL, pH 8). The two phases were separated, the or-

ganic layer was dried (Na_2SO_4) and filtered, and the solvent was removed in vacuo. Compounds **5a–d** were isolated by flash chromatography on silica gel (cyclohexane/EtOAc 8:2 as eluent).

Benzyl 3-[3-isopropyl-5-methyl-4-(4-nitrobenzylcarbamoyl) isoxazol-2(3*H*)-yl]-3-oxopropanoate (5b): Yellow oil (166 mg, 69%): ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.75 (d, J = 6.6 Hz, 3H), 0.84 (d, J = 6.6 Hz, 3H), 1.24 (m, 1H), 2.02 (s, 3H), 3.39 (d, J = 15.8 Hz, 1H), 3.61 (d, J = 15.8 Hz, 1H), 4.51 (d, J = 5.8 Hz, 2H), 5.07 (s,2H), 5.30 (bs, 1H), 6.67 (bt, 1H), 7.19 (m, 5H), 7.34 (d, J = 8.4 Hz, 2H), 8.11 ppm (d, J = 8.4 Hz, 2H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 10.9, 16.4, 19.1, 31.2, 43.5, 66.1, 67.6, 105.8, 122.9, 127.3, 127.5, 128.3, 128.7, 135.8, 140.9, 145.3, 156.9, 162.5, 166.0, 168.2 ppm; LC–MS (ESI): t_R = 10.0 min, m/z: 482 [M+1], 423 [M+Na].

General procedure for hydrogenation of 5a–d: To a solution of **5a–d** (0.5 mmol) in MeOH, Pd/C (10 mg) was added in one portion. The reaction mixture was stirred vigorously at room temperature under H_2 atmosphere overnight. The solution was filtered to remove catalyst, and the solvent was evaporated in vacuo. The crude compound was treated with Dowex 50WX2-200 ion-exchange resin, eluting with NH_4OH (0.5 M). Compounds **6a–d** were isolated after removal of the aqueous solvent in vacuo.

Benzyl 3-[3-isopropyl-5-methyl-4-(4-aminobenzylcarbamoyl) isoxazol-2(3*H*)-yl]-3-oxopropanoate (6b): Yellow oil (143 mg, 79%): ^{1}H NMR (200 MHz, CD_3OD): δ = 0.73 (d, J = 6.6 Hz, 3H), 0.86 (d, J = 6.6 Hz, 3H), 1.89 (m, 1H), 2.10 (s, 3H), 4.23 (d, J = 15.0 Hz, 1H), 4.35 (d, J = 15.0 Hz, 1H), 4.81 (s, 2H), 5.31 (bs, 1H), 7.00 (d, J = 8.0 Hz, 2H), 7.20 (d, J = 8.0 Hz, 2H), 8.21 ppm (bt, 1H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 11.4, 17.0, 20.1, 27.8, 30.7, 45.5, 69.8, 117.0, 120.7, 129.5, 129.7, 136.3, 146.3, 161.6, 163.4, 167.1 ppm; LC–MS (ESI): t_R = 1.46 min, m/z: 362 [M+1], 745 [2M+Na].

Coupling of 4b with *p*-aminobenzylamine (compound 7): HBTU (1.3 equiv, 0.65 mmol, 246 mg) and Et_3N (2.3 equiv, 1.15 mmol, 160 μL) were added to a stirred solution of acid **4b** (0.5 mmol) in a 1:1 mixture of dry CH_2Cl_2 (2 mL) and CH_3CN (2 mL), under N_2 atmosphere. After 30 min, *p*-aminobenzylamine (1.2 equiv, 0.6 mmol, 68 μL) was added, and the solution was stirred overnight. The mixture was diluted with CH_2Cl_2 (8 mL) and washed with acidic H_2O (2×5 mL, pH 3) and basic H_2O (2×5 mL, pH 8). The two phases were separated, the organic layer was dried (Na_2SO_4) and filtered, and the solvent was removed in vacuo. Compound **7** was used in the following step without further purification.

Coupling with *N*-Cbz-glycine: $SOCl_2$ (1.2 mmol, 2.4 equiv) was added dropwise to a stirred solution of *N*-Cbz-glycine (1.2 equiv, 0.6 mmol, 125 mg) in dry CH_2Cl_2 (5 mL) at 0 °C. After removing the ice bath, the solution was stirred for 30 min at room temperature, and then Et_3N (1.2 mmol, 2.4 equiv, 167 μL) and compound **7** (0.5 mmol) were added. The solution was left to stir overnight, and then the reaction mixture was diluted with CH_2Cl_2 (10 mL) and washed with H_2O (2×10 mL). The organic layer was dried (Na_2SO_4) and filtered, and the solvent was removed in vacuo. Purification of the crude residue by flash chromatography on silica gel (eluent cyclohexane/EtOAc 9:1) afforded compound **8**. Following hydrogenation and purification procedures reported above for **5**, compound **9** was isolated in nearly quantitative yield.

3-{4-[4-(2-Aminoacetamido)benzylcarbamoyl]-3-isopropyl-5-methylisoxazol-2(3*H*)-yl}-3-oxopropanoic acid (9): Yellow oil (410 mg, 98%): ^{1}H NMR (200 MHz, CD_3OD): δ = 0.78 (d, J = 6.6 Hz, 3H), 0.89 (d, J = 6.6 Hz, 3H), 1.96 (m, 1H), 2.07 (s, 3H), 3.31 (m, 2H), 3.52 (m, 2H), 4.23 (d, J = 15.6 Hz, 1H), 4.44 (d, J = 15.6 Hz, 1H), 5.40 (bs, 1H), 7.22 (d, J = 6.2 Hz, 2H), 7.43 ppm (d, J = 6.2 Hz, 2H); ^{13}C NMR (50 MHz, CD_3OD): δ = 11.3, 16.5, 19.8, 30.7, 33.4, 42.2, 43.5, 69.0, 107.5, 120.9, 129.2, 136.4, 138.1, 159.7, 165.3, 165.5, 167.9, 170.4 ppm; LC–MS (ESI): t_R = 0.83 min, m/z: 419 [M+1].

Biology

Cell culture: SK-MEL-24 cells (American Type Culture Collection, ATCC, Rockville, MD, USA) were routinely grown in minimum essential medium (MEM, Cambrex, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), nonessential amino acids, and sodium pyruvate. K562 cells (ATCC) were maintained as a stationary suspension culture in RPMI-1640 and L-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^{-1} PMA (Sigma–Aldrich SRL, Milan, Italy) to induce differentiation and to increase expression of cell surface antigens.[38]

Cell adhesion assays:[33] 96-well plates (Corning Costar, Celbio, Milan, Italy) were coated by passive adsorption with fibronectin (10 μg mL^{-1}) or poly-L-lysine (0.002%; Sigma–Aldrich SRL) overnight at 4 °C. Cells were counted with a hemocytometer and pre-incubated with various concentrations of each compound for 30 min at room temperature to reach a ligand–receptor equilibrium. Stock solutions (10–2 M) of the assayed compounds were prepared in phosphate-buffered saline (PBS). At the end of the incubation time, the cells were plated (50000 cells per well) and incubated at room temperature for 1 h. All the wells were then washed with PBS to remove nonadherent 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, the absorbance of which can be measured at λ 405 nm. As previously described,[39] there is a linear correlation between absorbance and enzymatic activity. Therefore, it is possible to identify the number of adherent cells among treated wells, interpolating the absorbance values of unknowns in a calibration curve. The reaction was blocked by the addition of 100 μL of a stopping solution [50 μM glycine and 5 μM EDTA (pH 10.4)], and the plates were read in a Victor2 Multilabel Counter (PerkinElmer, Waltham, MA, USA). Experiments were carried out in quadruplicate and repeated at least three times. Data analysis and IC_{50} values were calculated using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA).

Western blot analysis: K562 cells were incubated in RPMI-1640 with 1% FBS for 16 h. Plates were coated with fibronectin (10 μg mL^{-1}) and blocked with 1% BSA (Sigma–Aldrich SRL). Subsequently, 4×10^6 cells were pre-incubated with the various compounds for 30 min. Cells were allowed to adhere for 1 h on fibronectin in RPMI-1640 with 1% FBS. The cells were then lysed in mammalian protein extraction reagent (M-PER; Pierce, Rockford, IL, USA) supplemented with phosphatase inhibitor (Sigma–Aldrich SRL) for 10 min at 4 °C by gentle shaking. Cell debris was removed by centrifugation (14000 *g* for 15 min at 4 °C), and protein concentrations were estimated by BCA assay (Pierce, Rockford, IL, USA). Protein extracts (100 μg) were denatured at 95 °C for 3 min before loading and separation by 12% SDS-PAGE. The membranes were blocked in 1% BSA and incubated for 2 h with anti-phospho-ERK 1/2 (extracellular signal-regulated kinase 1/2) (1:2500) or anti-total ERK 1/2 antibodies (1:5000) (Promega, Madison, WI, USA), and thereafter with anti-rabbit peroxidase-conjugated secondary antibody. Digital

images were acquired and analyzed by following previously reported methods.[40]

Molecular docking

All calculations were run using the Schrödinger suite of programs (http://www.schrodinger.com) through the Maestro graphic interface.

Protein setup: The recently determined crystal structure of the extracellular domain of the integrin $\alpha_v\beta_3$ receptor in complex with cilengitide and in the presence of the pro-adhesive ion Mn^{2+} (PDB ID: 1L5G)[17] was used for docking studies. Docking was performed only on the globular head of the integrin because the head group of integrin has been identified in the X-ray structure as the ligand binding region. The protein structure was set up for docking as follows: the protein was truncated to residues 41–342 for chain α and residues 114–347 for chain β. Due to a lack of parameters, the Mn^{2+} ions in the experimental protein structure were modeled via replacement with Ca^{2+} ions. The resulting structure was prepared with the Protein Preparation Wizard of the graphical user interface Maestro and the OPLSAA force field.

Docking: Automated docking calculations were performed with Glide (Grid-based Ligand Docking with Energetics)[36] within the framework of Impact version 4.5 in a standard precision (SP) mode. The grid-generation step started from the extracellular fragment of the X-ray structure of the $\alpha_v\beta_3$ complex with cilengitide, prepared as described above in *Protein setup*. The center of the grid-enclosing box was defined by the center of the bound ligand. The enclosing box dimensions, which are automatically deduced from the ligand size, fit the entire active site. For the docking step, the size of the bounding box for placing the ligand center was set at 12 Å. No further modifications were applied to the default settings. The GlideScore function was used to select 20 poses for each ligand. Glide was initially tested for its ability to reproduce the crystallized binding geometry of cilengitide. The program was successful in reproducing the experimentally determined binding mode of this compound, as it corresponds to the best-scored pose.

Acknowledgements

This study was carried out with fundamental contributions of the "Fondazione del Monte di Bologna e Ravenna", MIUR (PRIN 2008) and the University of Bologna (Italy). We also thank the Italian Minister for Foreign Affairs (MAE) (General Direction for the Cultural Promotion and Cooperation) for financial support of a bilateral project between Italy and Mexico. Mr. Andrea Garelli is gratefully acknowledged for the LC–ESI-MS analysis. We also thank the Consorzio Interuniversitario Nazionale "Metodologie e Processi Innovativi di Sintesi" (CINMPIS) for a grant to A.V.

Keywords: cancer · cell adhesion · drug discovery · heterocycles · peptidomimetics

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Received: July 30, 2011
Revised: August 30, 2011
Published online on September 26, 2011

