Integrin Ligands

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# Breaking the Dogma of the Metal-Coordinating Carboxylate Group in Integrin Ligands: Introducing Hydroxamic Acids to the MIDAS To Tune Potency and Selectivity**

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The inhibition of cell adhesion by integrin ligands is a promising target for drug design. All integrins contain a metal-ion-dependent adhesion site (MIDAS), in which the metal ion is coordinated through five of six possible coordination sites. The extracellular matrix ligand provides the sixth binding site, for example the carboxyl group of an aspartic acid in the well-known RGD tripeptide sequence. So far all proteins and small peptidic and non-peptidic ligands have contained a carboxyl group for the metal-ion binding. All attempts to mimic this carboxyl group by "isosteric" groups have failed so far. Herein, we report that hydroxamic acids can be used successfully for this purpose, and that the binding affinity of the new ligands is retained or modulated. This is of special importance because the carboxyl group, which is ionized under neutral pH conditions, accounts for a strong barrier in the pharmaco-dynamic behavior of the integrin ligands. In contrast, hydroxamic acids are not ionized under the same conditions.

Integrins constitute a family of heterodimeric, transmembrane, bidirectional adhesion receptors, which connect cells to the scaffolding proteins of the extracellular matrix.[1] Disturbance of integrin function is connected to a large variety of pathological processes such as thrombosis,[2] cancer,[3] osteoporosis,[4] and inflammation,[5] which makes integrins attractive targets for pharmacological research. Of the 24 different heterodimers known, the integrins αvβ3, αvβ5, and α5β1 have attracted particular interest: They are key factors of angiogenesis (the formation and maturation of new blood vessels), a process that plays an important role in tumor progression and metastasis.[3,6] The natural ligands of the three integrins share the common tripeptidic recognition motif arginine–glycine–aspartate (RGD).[7] The fact that particular integrins are able to selectively bind different spatial presentations of one binding motif along with their great medical relevance has inspired researchers to design a vast number of different peptidic and non-peptidic integrin ligands.[8] As an example, the potent αvβ3 ligand, the cyclic peptide Cilengitide[9] (*cyclo*(RGDf*N*MeV)) is currently in phase III clinical trials for patients with glioblastoma multiforme, while the peptidomimetic αIIbβ3 binder Tirofiban[10] is an approved anticoagulant drug. However, the application of RGD-based drugs is hampered by their poor pharmacological properties, which may to some extent be the result of the zwitterionic nature of the RGD motif. 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 and through the synthesis of prodrugs.[11]

While the guanidine group of the arginine has been replaced by countless basic heterocycles during the development of peptidomimetics, the carboxylic acid function of the aspartate is the most conserved feature of all known integrin ligands up to now. Indeed, to our knowledge, the successful replacement of the carboxylic acid moiety has never been reported. The acid is involved in the crucial coordination of the bivalent metal cation at the MIDAS site, which is present in all integrins.[12] Although the metal ion has not yet been identified ($Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ are under discussion), the importance of the cation–carboxylate interaction is indisputable.[13] In our previous research we could demonstrate how the selectivity between the integrins α5β1 and αvβ3—the most important integrins in angiogenesis—can be switched in either direction by changing the ligand length and altering sterically demanding moieties close to the metal-coordination site.[14] Even though this site seems to be very sensitive towards modifications—several attempts to replace the carboxylate by tetrazole or sulfonic acids failed in the past—we thought about alternatives to a carboxylic acid that would lead to another binding mode and thus to an alteration in the selectivity profile.

We investigated hydroxamic acids, which seemed promising candidates as they can coordinate metals in a bi- or monodentate fashion depending on the environment, and in fact they are known to coordinate many different metal

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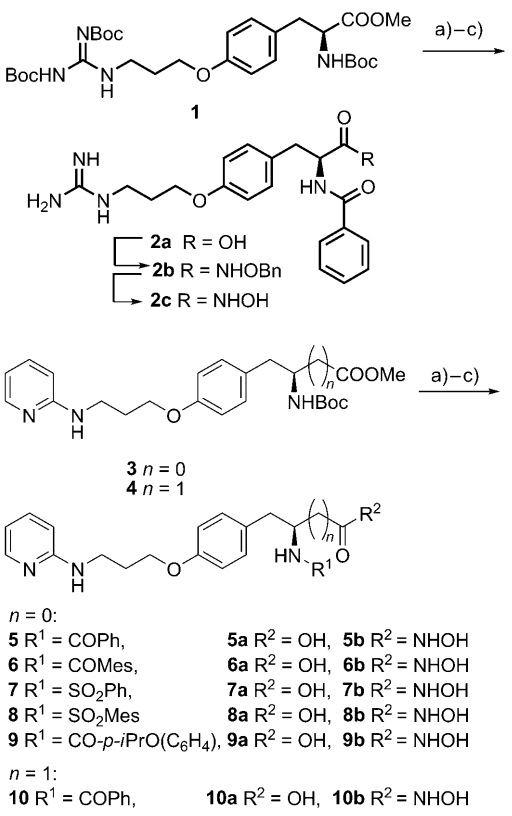
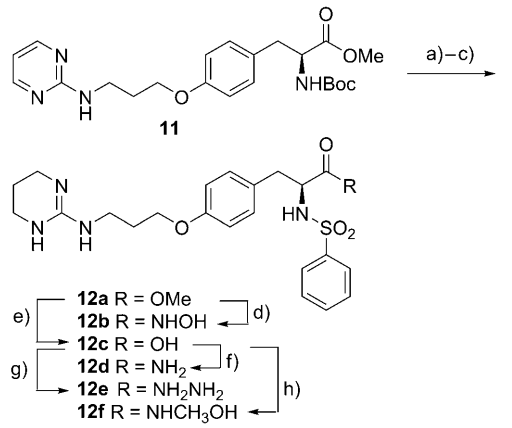
ions.[15] We first examined the ligands **2** (see Scheme 1) as proof of principle (**2a**: $IC_{50}$: 60 nM for α5β1 and 131 nM for αvβ3) and observed that for **2c** the $IC_{50}$ towards α5β1 increased to 6700 nM, while the corresponding value for αvβ3 decreased to 53 nM. These findings motivated us to systematically elucidate the potential of hydroxamic acids as integrin ligands as well as the structural and electronic aspects of the observed selectivity. In our previous studies we found that the spatial orientation of the aromatic moiety in the vicinity of the carboxylic acid determines the selectivity of the ligand; a mesitylene carboxamide unit led to α5β1 selectivity, while a sulfonamide group yielded biselective ligands.[14b–d] We were expecting that the replacement of the carboxylate by a hydroxamate should have a high impact on the positioning of this group, so six pairs of ligands, all containing a 2-aminopyridine group as the basic moiety, were synthesized and evaluated for their activity and selectivity profile. Furthermore, we prepared the hydroxamate analogue of an αvβ3 selective ligand based on an elongated β-homotyrosine. We also preapared another compound library based on a lead structure comprising a tetrahydropyrimidine as the basic moiety and a benzosulfonamide substituent, which was previously found to give ligands with high affinity for αvβ3 and moderate affinity for α5β1.[14b,c] Variations of the carboxylic acid function including an ester, amide, acylhydrazine, and *N*-methyl hydroxamic acid should reveal, whether other carboxylic acid derivatives lead to changes in affinity and selectivity comparable to hydroxamic acids.

The synthesis of all the ligands started from known precursors (**1**, **3**, **4**, and **11**).[14b,c] After Boc removal with diluted aqueous HCl in dioxane, the resulting amines were acylated with either aromatic carboxylic acids or aromatic sulfonyl chlorides according to the desired selectivity profile. While saponification of the methyl ester with LiOH in methanol/water gave the carboxylic acids, a feasible way to prepare the corresponding hydroxamic acids was the addition of an excess of hydroxylamine to the saponification mixture.[16] A previously examined procedure, the KCN-catalyzed aminolysis of methyl esters (**11**→**12b**; see Scheme 2), was abandoned because of lower yields and longer reaction times. In contrast to the other hydroxamic acid ligands, **2b** was prepared by coupling of the free acid to *O*-benzylhydroxylamine followed by hydrogenolysis. This reaction was found to be difficult to control as sometimes overreduction to the amide takes place, and it was therefore also not applied in the synthesis of other hydroxamate ligands (Scheme 1). The second series of ligands started from precursor **11**, which was transformed into the derivatives **12a**–**f** (Scheme 2). All ligands were purified by reverse-phase HPLC and evaluated in an enzyme-linked immunosorbent assay (ELISA) using the immobilized natural integrin ligands fibronectin and vitronectin and the soluble integrins α5β1 and αvβ3, respectively.

Computational studies were performed to understand the selectivity of the inhibition of αvβ3 or α5β1 integrin receptors by ligands **5**–**9**. Table 1 shows that the different metal-coordinating groups together with the bulky substituent in the α position are the major determinants for the inhibitory activity as well as for the receptor selectivity. Thus, to address this issue, the inhibitors were automatically docked, with the



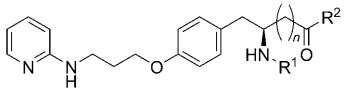

***Scheme 1.*** Synthesis of hydroxamic acid and carboxylic acid ligands **5**–**10**. Reagents and conditions: a) $HCl/H_2O$/dioxane; then b) PhCOCl, $NaHCO_3$, $THF/H_2O$, or MesCOOH, HATU, DIPEA, DMF, or $ArSO_2Cl$, DIPEA, DMF; c) LiOH, $MeOH/H_2O$ (acids) or LiOH, $NH_2OH$ (aq.), $MeOH/H_2O$ (hydroxamates). All compounds were purified by RP-HPLC using $MeCN/H_2O$ + 0.1% TFA as eluent. For reaction details and analytical data see the Supporting Information. Bn = benzyl, Boc = *tert*-butoxycarbonyl, DIPEA = diisopropylethylamine, HATU = 2-(1*H*-7-azabenzotriazo-1-yl)-1,1,3,3-tetramethyluronium hexaphosphate, Mes = 2,4,6-trimethylphenyl, TFA = trifluoroacetic acid.

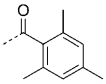
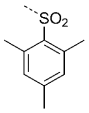
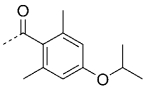



***Scheme 2.*** Synthesis of ligands **12a**–**f**. Reagents and conditions: a) HCl/dioxane; b) $PhSO_2Cl$, DIPEA, DMF; c) $H_2$/Pd/C, MeOH; d) KCN, $NH_2OH$, $MeOH/H_2O$; e) LiOH, $MeOH/H_2O$; f) Rink amide resin, TBTU, HOBt, DIPEA, NMP, then 95% TFA; g) $NH_2NHBoc$, TBTU/HOBt, DMF; h) LiOH, NHMeOH, $MeOH/H_2O$. For reaction details and analytical data see the Supporting Information. HOBt = 1-hydroxy-1*H*-benzotriazole, NMP = *N*-methylpyrrolidinone, TBTU = 2-(1*H*-benzotriazo-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

***Table 1:*** $IC_{50}$ values of integrin ligands for α5β1 and αvβ3.



| Cmpd | $R^2$ | *n* | $R^1$ | $IC_{50}$ [nM] α5β1[a] | $IC_{50}$ [nM] αvβ3[a] |
|---|---|---|---|---|---|
| **5a** | -OH | 0 |  | 243 | 207 |
| **5b** | -NHOH | | | 2470 | 14 |
| **6a** | -OH | 0 |  | 2.5 | 703 |
| **6b** | -NHOH | | | 1244 | 72 |
| **7a** | -OH | 0 |  | 284 | 1.9 |
| **7b** | -NHOH | | | 296 | 11 |
| **8a** | -OH | 0 |  | 46 | 3.4 |
| **8b** | -NHOH | | | 132 | 4.8 |
| **9a** | -OH | 0 |  | 1 | 279 |
| **9b** | -NHOH | | | 40 | 13.5 |
| **10a** | -OH | 1 |  | 264 | 1.2 |
| **10b** | -NHOH | | | 4500 | 12 |

[a] $IC_{50}$ values were derived from competitive ELISA using the immobilized natural integrin ligands fibronectin and vitronectin and the soluble integrins α5β1 and αvβ3, respectively (for details see the Supporting Information).

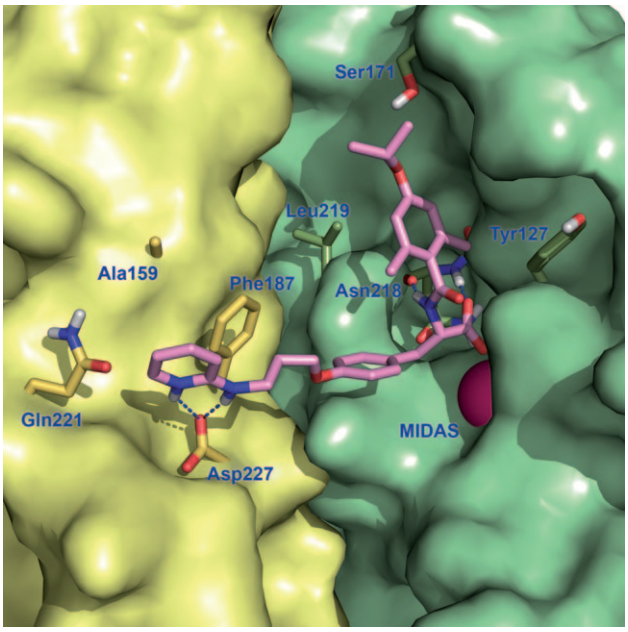



***Figure 1.*** Structure of **9a** (pink) docked in the α5β1 integrin binding pocket. The α5 and β1 subunits are represented by the yellow and green surfaces, respectively. In both subunits the amino acid side chains important for the ligand binding are represented as sticks. The metal ion in the MIDAS region is represented by a magenta sphere.

aid of AutoDock4 (AD4), in our published homology model of the α5β1 integrin[14a] and in the X-ray structure of the αvβ3 receptor in complex with Cilengitide (PDB code: 1L5G)[17] after removal of the cocrystallized inhibitor. As the docking results using AD4 with the default charges (Gasteiger) on the ligands and the protein were not entirely satisfactory in reproducing the coordination geometry of the metal-coordinating groups, we performed preliminary ab initio calculations on the manganese ion in the MIDAS region, on its coordinating amino acids (Mn subsite), and on the ligands themselves (see the Supporting Information for details). The charges obtained through these calculations were used to give a more accurate reproduction of the experimental binding geometry of Cilengitide, and this was a prerequisite for the docking of compounds **5**–**9**.

According to our docking results, compound **9a** coordinates the metal ion in integrin α5β1 with one of the two oxygens of the carboxylate group, while the other forms a hydrogen bond with the backbone NH group of (β1)-Asn218; this coordination mode is similar that of the Cilengitide carboxylic group in the X-ray crystal structure (Figure 1). The isopropyloxyphenyl moiety fits well in the β1 region where it is involved in a π–π interaction with (β1)-Tyr127 (distance between the centroids of the rings: 6.1 Å). In this arrangement, the *p*-isopropyloxy group is in proximity to the (β1)-Ser171 side chain (distance between the two oxygens: 3.6 Å), which protrudes from the (β1)-SDL (specificity-determining loop), and a hydrogen bond is likely formed. The tyrosine scaffold of **9a** was found in proximity to (α5)-Phe187, allowing the basic moiety to form a bifurcated salt bridge with the highly conserved (α5)-Asp227. Clearly, all of these interactions are responsible for the subnanomolar activity of **9a** towards the α5β1 receptor, while its decreased affinity (1000-fold) for αvβ3 has been previously attributed by us to the steric clashes between the isopropyloxyphenyl moiety of **9a** and the (β3)-Arg214 side chain.[14b,c]

Interestingly, in the docking the hydroxamic acid analogue **9b** into αvβ3, either a bidentate (*O*,*O*)-chelating mode or a monodentate (*O*)-coordination mode were found. However, unexpectedly, the bidentate (*O*,*O*)-chelating mode, which is most commonly observed in biological systems, is rarely found in our docking study and did not result in any reasonable binding mode. Indeed, if a bidentate (*O*,*O*)-chelating mode is considered, owing to the shape of the binding site and the presence of (β3)-Ser121, (β3)-Glu220, and (β3)-Ser123, which directly coordinate the metal in the MIDAS, the basic moiety of the ligand cannot be properly inserted into the narrow groove at the top of the propeller domain of αv, where contacts with (β3)-Asp218 and/or (β3)-Asp150 are expected to occur. In contrast, one of the monodentate coordination modes calculated by the AD4 program, was highly meaningful; it positioned the ligand in a proper manner to form a π–π interaction with the (αv)-Tyr178 (through its tyrosine scaffold), a hydrophobic interaction with the (β3)-Tyr122 through the isopropyloxyphenyl moiety, and also a bifurcated salt bridge between the basic moiety and the (αv)-Asp218, in addition to the coordination of the metal in the MIDAS region (Figure 2).

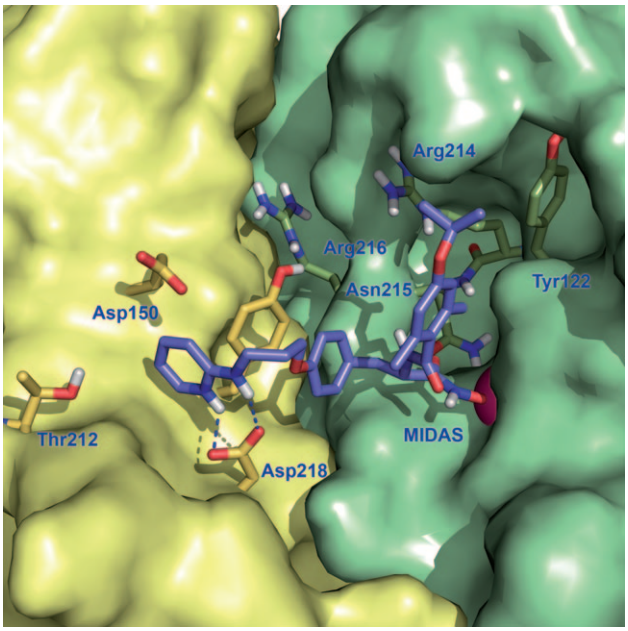



***Figure 2.*** Structure of structure of **9b** (blue) docked in the αvβ3 integrin binding pocket. The αv and β3 subunits are represented by the yellow and green surfaces, respectively. In both subunits the amino acid side chains important for the ligand binding are represented as sticks. The metal ion in the MIDAS region is represented by a magenta sphere.

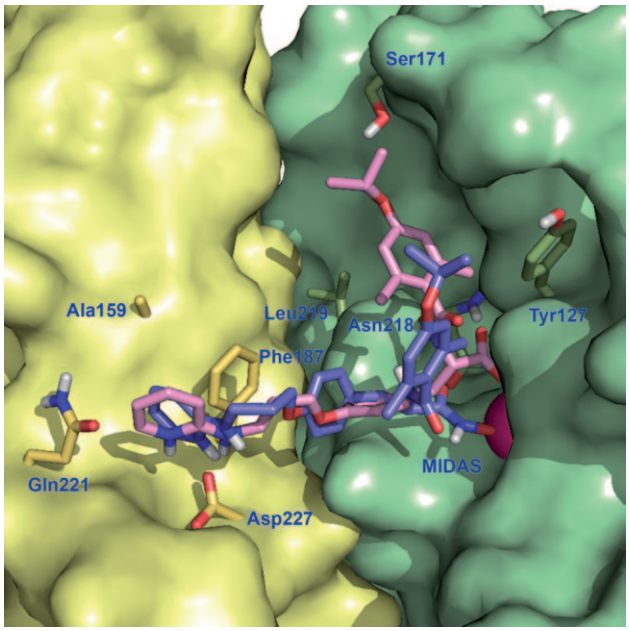



***Figure 3.*** Superposition of the binding mode of **9b** (blue) docked in αvβ3 and that of **9a** (pink) docked in α5β1. For clarity only the surface of α5β1 is shown.

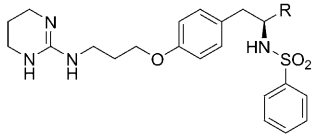
The results shown in Table 1 outline that in **9a** the replacement of the carboxylic group by the hydroxamate moiety allows **9b** to regain the activity for αvβ3 receptor. If the binding mode observed for **9a** (in α5β1, Figure 1) is superimposed on that of **9b** (in αvβ3, Figure 2), a down-shifting of the isopropyloxyphenyl moiety is observed for the hydroxamate derivative (Figure 3), which would account for the activity for αvβ3. Moreover, as a result of the structural difference between the carboxylic and the hydroxamic acids (the latter has a larger distance between the two oxygen atoms), the distance between the metal-coordinating oxygen and the bulky substituent in the α position is greater in **9b** than in **9a**. As a consequence, the coordination made by the hydroxamate compound **9b** allows a shifting of the isopropyloxyphenyl moiety towards the α subunit and an orientation that allows the isopropyloxyphenyl group to form hydrophobic interactions with (β3)-Tyr122 (Figure 3). Another intriguing point is the inverted selectivity of compound **9b** with respect to **9a**. In fact, compound **9b** slightly prefers inhibiting the αvβ3 receptor over the α5β1 receptor. According to our results, this is a consequence of the increased distance between the acidic and the basic groups, as a result of the presence of the hydroxamate moiety in **9b** (see above). In fact, in line with our recently published results,[14b,c] the mutation of (αv)-Thr212 to (α5)-Gln221 in the α5β1 receptor reduces the space available for the binding of the ligand's basic moiety, and, consequently, compounds with shorter chains are preferred to bind to the α5β1 receptor. Accordingly, compound **10b**, whose length is increased by one methylene group in addition to the hydroxamate, additionally, shows no activity for α5β1. Concerning compound **8a**, previous docking experiments indicated that the presence of the sulfonamide allows the mesitylene group to fold back towards the α subunit endowing the ligand an inhibitory activity toward both receptors. The slightly higher activity of **8a** for αvβ3 can be attributed to polar contacts between the sulfonamide oxygens and the guanidine group of (β3)-Arg214. Consequently, the hydroxamate analogue **8b** has a lower affinity for the α5β1 integrin than its carboxylate analogue **8a**, and the reason again seems to reside in the greater distance between the metal-coordinating oxygen and the basic moiety. Regarding the selectivity profile of compound **8b**, an issue similar to that of compound **9b** can be assumed.

To investigate whether other derivatives of carboxylic acids and hydroxamic acids are capable of integrin binding, we compared six different C termini of one ligand, which was supposed to display high αvβ3 activity in its carboxylic acid form **12c**. Table 2 shows the outstanding high affinity of the hydroxamic acid **12b** in contrast to the other derivatives. Remarkably, despite their reduced acidity [($pK_a$(*N*-hydroxyacetamide) = 9.40 compared to acetic acid (4.76)],[18] the hydroxamates are still able to complex the metal ion efficiently. The low affinity of **12e** is the result of the replacement of the MIDAS-binding oxygen by a hydrazone $NH_2$ group with poor coordination properties; an even more drastic effect can be observed for the amide **12d**. Similar to **12e**, residual binding affinity can still be observed for the methyl ester **12a**. The sensitivity of the binding mode towards additional substituents is demonstrated by the low affinity of the *N*-methylated hydroxamic acid **12f**.

Based on a homology model of the integrin α5β1 and previous studies on the structure–activity relationship, we report the first replacement of the ubiquitous carboxylic acid function in integrin ligands. Extensive modeling of the

***Table 2:*** $IC_{50}$ values of integrin ligands for α5β1 and αvβ3.



| Cmpd | R | $IC_{50}$ [nM] α5β1[a] | $IC_{50}$ [nM] αvβ3[a] |
|---|---|---|---|
| **12a** | -COOMe | 2366 | 419 |
| **12b** | -CONHOH | 85 | 5.3 |
| **12c** | -COOH | 79 | 4.2 |
| **12d** | $-CONH_2$ | >20000 | >1000 |
| **12e** | $-CONHNH_2$ | 9000 | 290 |
| **12f** | $-CONCH_3OH$ | 5216 | 359 |

[a] $IC_{50}$ values were derived from competitive ELISA using the immobilized natural integrin ligands fibronectin and vitronectin and the soluble integrins α5β1 and αvβ3, respectively (for details see the Supporting Information).

MIDAS region of αvβ3 and α5β1 helped to determine the binding mode of this new class of ligands and to rationalize the observed selectivities for the integrin αvβ3. Our findings break with the dogma of carboxylic acid functionalized RGD mimetics and may yield novel lead structures for pharmaceutical research.

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**Keywords:** antitumor agents · hydroxamic acids · integrin ligands · molecular modeling · structure–activity relationship

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