Small Molecule Designed to Target Metal Binding Site in the α2I Domain Inhibits Integrin Function

Jarmo Käpylä,[†,‡] Olli T. Pentikäinen,[†,§,||] Tommi Nyrönen,[⊥] Liisa Nissinen,[#] Sanna Lassander,[‡] Johanna Jokinen,[‡] Matti Lahti,[‡] Anne Marjamäki,[#] Mark S. Johnson,[⊗] and Jyrki Heino*[,‡]

Department of Biochemistry and Food Chemistry, University of Turku, FI-20014 Turku, Finland, Fatman Bioinformational Designs Ltd., FI-20520 Turku, Finland, Finnish IT Centre for Science, CSC, FI-02101 Espoo, Finland, BioTie Therapies Ltd., FI-20520 Turku, Finland, and Department of Biochemistry and Pharmacy, Åbo Akademi University, FI-20520 Turku, Finland

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Integrin α2β1 is a potential target molecule in drug development. We have established "design" criteria for molecules that bind to the "closed" conformation of α2I domain via Mg^{2+} in MIDAS (metal ion dependent adhesion site) while simultaneously forming interactions with neighboring amino acid residues. Specific tetracyclic *Streptomyces* products belonging to the group of aromatic polyketides fulfill our criteria and inhibit α2β1 integrin. All previously described inhibitors of αI domain integrins act in an allosteric manner.

Introduction

Nine human integrins, four collagen receptors, and five leukocyte integrins recognize their ligands with a specific "inserted" domain (αI domain or αA domain) in their α subunit.[1,2] Integrin αI domains have a typical Rossman fold with a metal ion coordination site (MIDAS[a]). In the αI domain integrins, the natural ligands are in direct contact with Mg^{2+} coordinated by residues forming MIDAS in the αI domain.[1,2] Integrin αI domains have a very dynamic structure, especially during the ligand-induced integrin activation when conformation changes from a "closed" to an "open" one.[3] The αI domains of αLβ2 and αMβ2 appear to have a third "intermediate" conformational state.[4,5] The "closed", "intermediate", and "open" conformations have, respectively, increasing affinity for ligands.[4,5] Most of the drugs in clinical practice and drug trials that target integrins are antibodies, but there is an increasing interest to develop small molecule inhibitors.[6]

The structural dynamics occurring at the αI domains have made it difficult to design small molecule inhibitors that target MIDAS. Instead, the known small molecule inhibitors are allosteric inhibitors,[6,7] which either bind to the C-terminal α helix, in the case of the αLI domain, and stabilize the low affinity closed conformation (e.g., BIRT0377[8] and statins[9]) or bind to the β2I domain and modify the interface between the α and β2 subunits (e.g., compounds **1**, **3**, and **4**[10] and XVA143[11]). Molecules binding to β2 lack specificity and are inhibitors of all four β2 associated αI domain integrins (αLβ2, αMβ2, αXβ2, and αDβ2).[7] It was recently reported that arylamide derivatives can bind to α2I domain at a site corresponding to the statin binding site in αLI domain and act as allosteric inhibitors of α2β1 integrin.[12]

Results and Discussion

The design of small molecule inhibitors for the collagen receptor subgroup of the αI domain integrins (α1β1, α2β1, α10β1, and α11β1) faces the same problems seen for drug development targeted to the β2 integrins. Consequently, it has been uncertain whether it is possible to design small molecules that would attach to the same binding site recognized by the natural ligands, such as collagen molecules, providing opportunities to design integrin-specific ligands that take advantage of sequence variation found near MIDAS. The collagen binding surfaces on the α1I and α2I domains are relatively large, flat and small molecules cannot physically cover the entire binding area. Furthermore, the transformation from "closed" into the "open" conformation (Figure 1a,b) subsequently alters the structure of MIDAS (Figure 1a,b).

The most important structural changes relevant to the transition between the "open" and "closed" conformations are (1) the unwinding of the αC helix (284G-Y-L-N-R288), followed by (2) a formation of an additional turn in the α6 helix (Figure 1a,b and ref 3) and (3) consequent changes in the coordination of Mg^{2+} at MIDAS.[3] Based on these observations, we set two main principles that would describe optimal inhibitor ligands: (1) the inhibitor molecule should form constructive interactions with the αC helix and with amino acids situated just before the following helix, α6 (Figure 1a−c: orange surface), and (2) coordinate to the metal ion, Mg^{2+}, (Figure 1: yellow sphere) such that the coordination seen at MIDAS in the "closed" conformation of the αI domain is maintained.

To identify ligand frameworks, molecules from commercially available and academic collections were docked into the "closed" conformation of the α2I domain. Specific tetracyclic aromatic compounds, such as certain *Streptomyces* products belonging to the group of aromatic polyketides,[13] were found to fulfill best our set of structural criteria for binding as well as in filling the volume of the cavity above the MIDAS surface (Figure 1c−e). In docking simulations, these compounds are able to coordinate to the metal ion (Mg^{2+}; Figure 1c,d), the aromatic rings in the tetracyclic polyketides interact with Tyr285 at the root of the αC helix, and the polar substituents in the tetracyclic ring form favorable interactions with the main-chain amino group of Glu256 (Figure 1c−e). We predicted that these interactions would stabilize the positioning of the small molecule and correspondingly the "closed" α2I domain structure (Figure 1 a,c−e). As Leu296 is critical for binding these compounds, it is possible that these tetracyclic polyketides coordinate to the metal ion only via water molecule and rather stabilize the ligand position by using hydrophobic interactions with Leu296 (Figure 1e). In the first series of experiments, one compound called **4**

* To whom correspondence should be addressed. Telephone: +358 2 333 6879. Fax: +358 2 333 6860. E-mail: jyrki.heino@utu.fi.
[†] Equal contribution.
[‡] University of Turku.
[§] Fatman Bioinformational Designs Ltd.
[||] Present address: Department of Biological and Environmental Science, FI-40014 University of Jyväskylä, Finland.
[⊥] Finnish IT Centre for Science.
[#] BioTie Therapies Ltd.
[⊗] Åbo Akademi University.
[a] Abbreviations: MIDAS, metal ion dependent adhesion site; CHO, Chinese hamster ovary.

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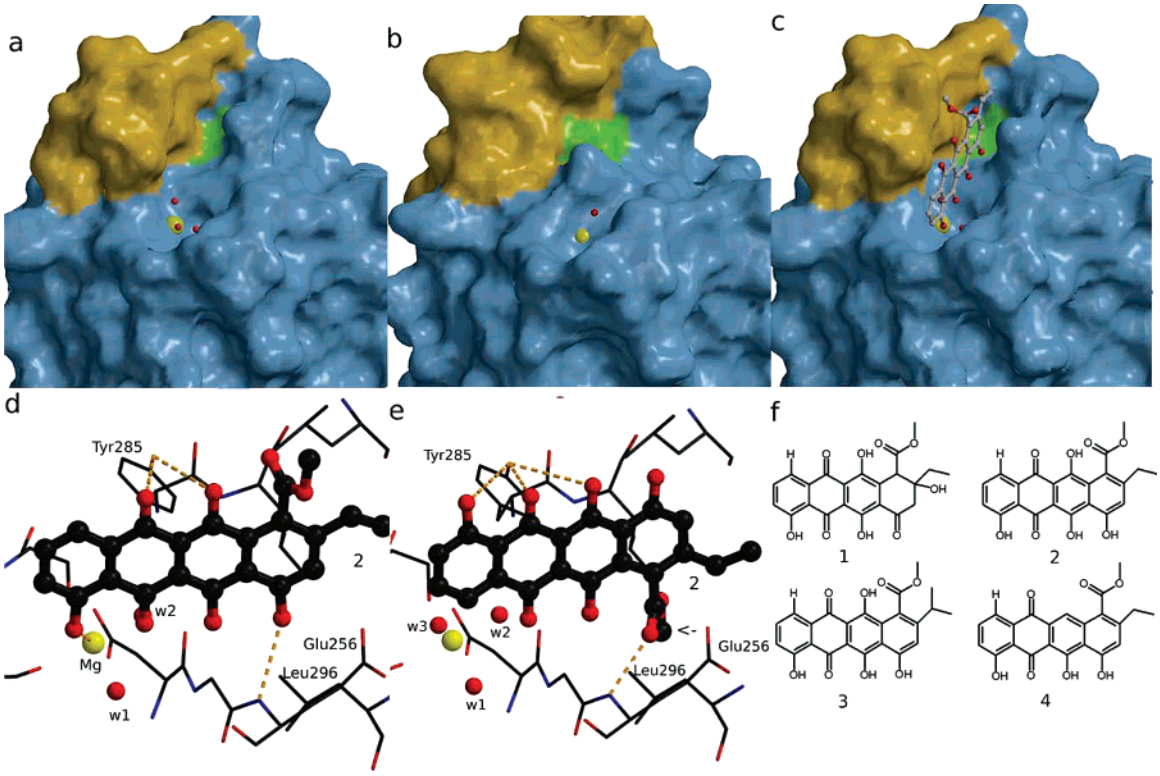

Figure 1. MIDAS in (a) the "closed" conformation and (b) "open" conformation (the bound collagen mimetic peptide is not shown). (c) Proposed binding of **2** into "closed" conformation of α2I domain fills the cavity at the MIDAS. (d,e) Detailed interactions of **2** for two alternative conformations in which the tetracyclic aromatic polyketides could bind to the closed conformation of the integrin αI domains. (f) Structures of the studied ligands. Compounds **1** and **2** are also known as maggiemycin and anhydromaggiemycin, respectively. Surface coloring (a−c): Gly284−Asp192, brown; Leu296, green; metal ion, yellow sphere; water molecules coordinated to the metal ion, red spheres. Possible hydrogen bonds between **2** and the integrin α2I domain are shown with orange dotted lines (d,e).

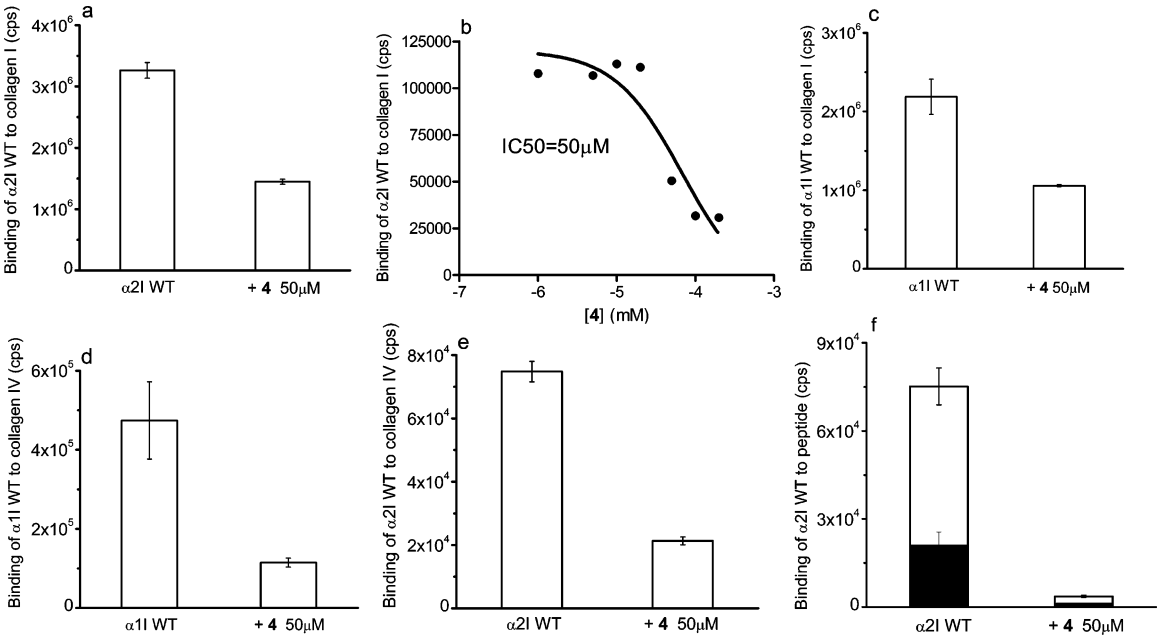

Figure 2. The effect of **4** on the binding of α1I and α2I to collagen I and IV. (a,c−e) Binding of α1I and α2I to collagen I and IV in the presence of 50 μM **4**. (b) Binding of α2I as a function of **4** concentration. (f) Binding of α2I to jararhagin (snake venom toxin) derived CTRKKHDNAQC− peptide in the presence of 50 μM **4**. Time-resolved fluorescence measurements were used. The data are means of three parallel determinations (±SD).

(methyl 2-ethyl-4,5,7-trihydroxy-6,11-dioxonaphthacenecarboxylate) inhibited α2I domain binding to collagen I (Figure 2a). It inhibited about 85% of total binding with an IC_{50} value of about 50 μM (Figure 2b). Importantly, in the same conditions, other highly similar tetracyclic polyketides had no effect (not shown), indicating that integrin binding is not a general property in this group of compounds, but that specific molecular features are needed for the recognition. The effect of **4** was not specific to the α2I domain, because it could also inhibit the binding of α1I (Figure 2c), and neither was the effect of **4** selective for collagen I, because it could also inhibit the binding of α1I (Figure 2d) and α2I (Figure 2e) to collagen IV. We have

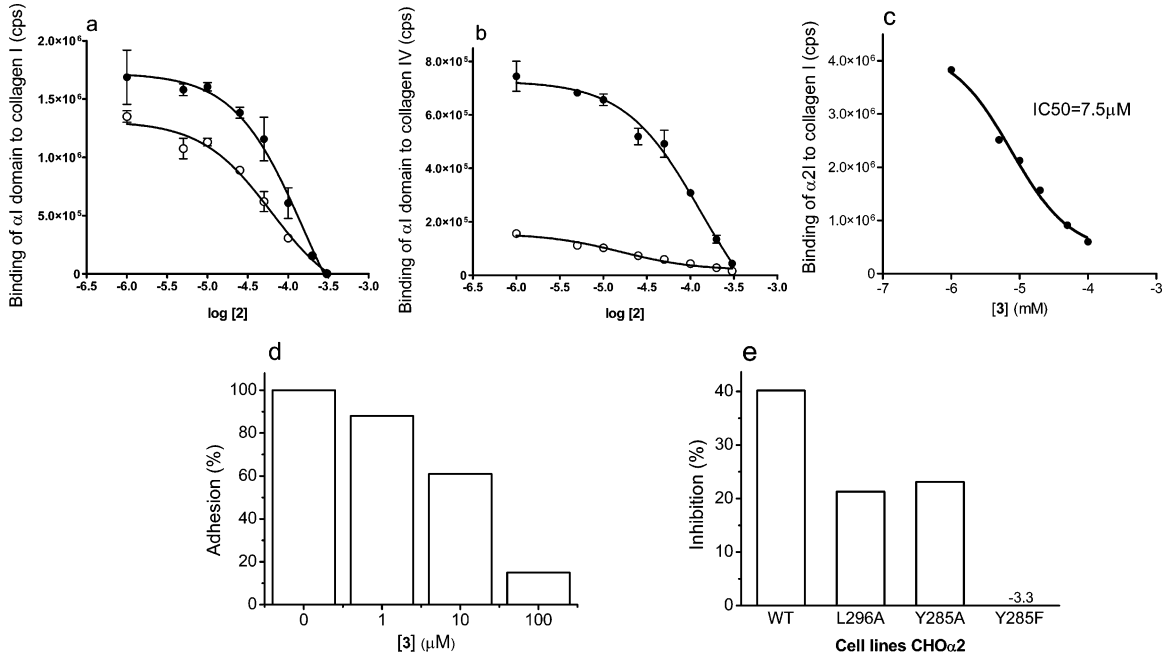

Figure 3. Binding of α2I (open circles) and α2IE318W (closed circles) to collagen I (a) as a function of **2** concentration. (b) Binding of α2I (open circles) and α2IE318W (closed circles) to collagen IV as a function of **2** concentration. Europium-labeled anti-GST assay. The data are means of three parallel determinations (±SD). (c) Binding of α2I to collagen I as a function of **3** concentration. Europium-labeled anti-GST assay. (d) Adhesion of CHO−α2β1 cells to collagen I as a function of **3** concentration. Cells were suspended in serum-free medium containing 0.1 mg/mL cycloheximide, and the compounds were preincubated with the cells prior to transfer to the wells. Cells were allowed to attach on collagen-type I coated wells for 2 h at +37 °C and after that nonadherent cells were removed. Fresh serum-free medium was added, and the living cells were detected. (e) Adhesion of CHO−α2β1 mutants to collagen I in the presence of **2**. Cells were suspended in serum-free medium containing 0.1 mg/mL cycloheximide, and the compounds were preincubated with the cells prior to transfer to the wells. Cells were allowed to attach on collagen I coated wells for 2 h at +37 °C and, after that, nonadherent cells were removed. Fresh serum-free medium was added, and the living cells were detected.

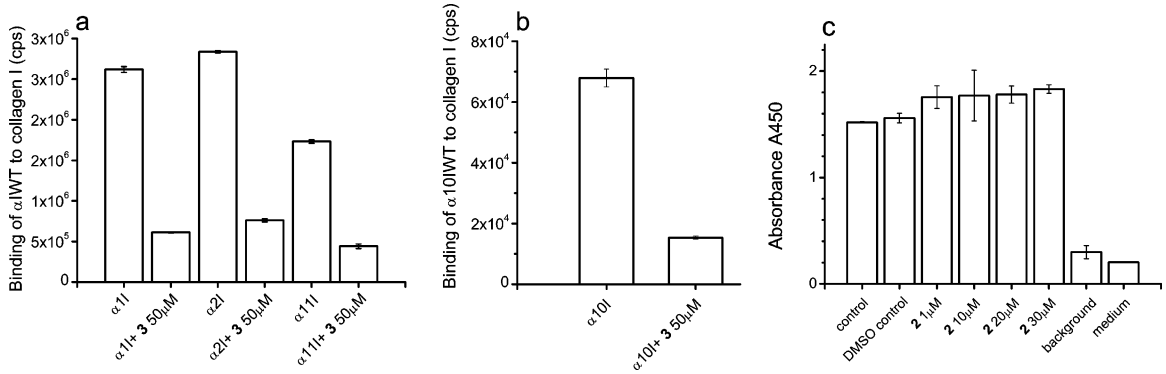

Figure 4. Binding of α1I, α2I, α10I, and α11I to collagen I in the presence of **3** (a,b). Binding of α1I, α2I, α10I, and α11I to collagen I in the presence of **3**. Europium-labeled anti-GST assay. Time-resolved fluorescence measurements were used. The data are means of three parallel determinations (±SD). (c) CHO cell adhesion on fibronectin in the presence and absence of **2**. Wells were coated with fibronectin (5 μg/cm2) and unspecific binding sites were blocked with 1% BSA in PBS. Cells were suspended in serum-free medium containing 0.1 mg/mL cycloheximide. Compound **2** was preincubated with the cells prior to transfer to the wells, and cells were allowed to attach on fibronectin for 2 h at +37 °C. After that, nonadherent cells were removed and fresh serum-free medium was added, and the living cells were detected. Background indicates the adhesion of the cells to only BSA-coated wells.

previously described a cyclic CTRKKHDNAQC-peptide[14] proposed to bind to the MIDAS surface of α1I and α2I domains.[15,16] Compound **4** could inhibit the binding of α2I domain to this peptide, suggesting that **4** binds to the MIDAS surface on α2I (Figure 2f). Under further analysis, the isolated preparation from biofermentation, compound **4**, turned out to contain smaller amounts of other highly similar compounds as impurities, too. After purification, these compounds, **1**−**3** (**1**, methyl 2-ethyl-2,5,7,12-tetrahydroxy-4,6,11-trioxo-1,2,3-trihydronaphthacenecarboxylate; **2**, methyl 2-ethyl-4,5,7,12-tetrahydroxy-6,11-dioxonaphthacenecarboxylate; **3**, methyl 4,5,7,-12-tetrahydroxy-2(methylethyl)-6,11-dioxonaphthacenecarboxylate) (Figure 1f), were isolated and tested. The most potent inhibitor was **3**, which had an IC_{50} value of 7.5−12 μM (Figure 3c) for the α2I domain, the IC_{50} of **2** was about 50 μM (Figure 3a), and compound **1** was clearly less effective (Table 1). Compounds **1** and **2** have been previously named maggiemycin and anhydromaggiemycin, respectively, and are described as potential antitumor agents.[17] To examine the role of the conformation of the α2I domain in the inhibition by compound **2**, the effect of **2** was tested with the "closed" conformation of the α2I domain (wild-type) and with "open" conformation of the α2I domain having E318W mutation. Mutation from glutamate to tryptophan in residue 318 is known to lead to a conformation resembling the conformation seen in the crystal structure with bound triple-helical collagen-like peptide.[3,18] The

Table 1. Maximal Inhibition in α2I Domain Binding Assays and Cell Adhesion Assays, Detected Cytoxicity, COL I, Collagen I

cmpd	maximal inhibition of α2I domain binding to COL I (%)	inhibition of cell adhesion to COL I (%; 10 μM)	cytotoxicity (0−200 μM)
1	65	10	not toxic ≤ 200
2	100	35	not toxic ≤ 50
3	90	40	not toxic ≤ 50
5	0	30	not toxic ≤ 200

"open" conformation of α2I domain showed increased binding to collagen I, and an even more pronounced change could be seen in the collagen IV binding (Figure 3a,b). The E318W mutation seemed to weaken the inhibitory effect of **2**: in the case of collagen I, the estimated IC_{50} values increased from 62 μM ("closed") to 160 μM ("open"), and in the case of collagen IV, the estimated IC_{50} values increased from 16 to 131 μM (Figure 3a,b). Thus, **2** could inhibit both α2I domain variants, but the "open" conformation seems to be less suitable for the binding of the inhibitor.

Integrin α2β1 can have several active conformations when expressed on the cell surface.[19,20] Because the compounds were selected based on their ability to inhibit the binding of the "closed" conformation of the α2I domain, it was critical for our hypothesis to test whether they had activity in cell-based assays. For this purpose, CHO cells were transfected to express α2β1 as their only collagen receptor, and cell adhesion assays were performed. Compounds **2** and **3** inhibited cell adhesion to collagen I when concentrations larger than 10 μM were used (Figure 3d, Table 1).

To confirm that the compounds bind to the α2I domain in the proposed manner, we mutated two amino acid residues, Tyr285 and Leu296, in the α2 cDNA expressed in CHO cells (Figure 1d,e). CHO cells harboring the mutations Y285A and L296A in the α2I domains could bind to collagen I, but **2** could no longer inhibit the binding (Figure 3e). According to the modeled complexes, when Tyr285 is mutated to alanine or phenylalanine, stabilizing hydrogen-bonding interactions with **2** would be lost and, thus, the inhibition of collagen binding is reduced (Figure 3e).

The selected aromatic polyketides also inhibited the other collagen binding αI domains, namely, α1I, α10I, and α11I (Figure 4a,b). Despite their structural differences, the four αI domains seem to bind to collagen using a similar basic mechanism. This is consistent with the fact that they all recognize similar binding motifs on collagens.[21] Tyr285, indicated to be a key residue for interactions with **2**, is conserved in α11I, whereas in α1I, it is a serine and in α10I, it is a histidine. Both serine and histidine have polar groups that can stabilize ligand binding through hydrogen-bonding interactions. The other key residue, Leu296, is not conserved either. In the α1I and α10I domains, phenylalanine is present at this position. The aromatic side chain of phenylalanine can serve in a similar way to the leucine side chain and pack against the hydrophobic face of the compounds. When Leu296 is mutated to the much smaller alanine, the binding and subsequent inhibition of collagen binding by the compounds is reduced (Figure 3e). The smaller alanine can provide space, for example, for a water molecule, resulting in an unfavorable interaction between water and alanine and between water and the hydrophobic methyl group of the ligand. Despite the fact that the selected aromatic polyketides inhibited all collagen receptor integrins, **2** had no effect on CHO cell adhesion to fibronectin (Figure 4c). This interaction is mediated by α5β1 and αV integrins. Thus, the compound seems to be specific for the collagen receptor subgroup of integrins.

Conclusions

The αI domain containing integrins are important targets in the development of drugs against thrombosis, inflammatory diseases, and cancer.[6,7] An intensive search for inhibitor molecules has led to the discovery of allosteric antagonists.[6,7] Here we have used the collagen receptor α2β1 and a set of structural criteria that molecules that could directly bind to MIDAS need to fulfill, which would result in inhibition of the ligand binding to the αI domain and probably stabilize the inactive, "closed" state of the receptor. We have identified specific aromatic tetracyclic polyketides and shown that this strategy can be successfully used. Other compounds may fulfill the same structural criteria as well. Our results are thus useful for further development of more selective or higher affinity inhibitors of the αI domain integrins.

Experimental Section

Tetracycline Compounds. A library of tetracycline compounds, including **1** (methyl 2-ethyl-2,5,7,12-tetrahydroxy-4,6,11-trioxo-1,2,3-trihydronaphthacenecarboxylate; also known as maggiemycin[17]), **2** (methyl 2-ethyl-4,5,7,12-tetrahydroxy-6,11-dioxonaphthacenecarboxylate, also known as anhydromaggiemycin[17]), **3** (methyl 4,5,7,12-tetrahydroxy-2(methylethyl)-6,11-dioxonaphthacenecarboxylate), **4** (methyl 2-ethyl-4,5,7-trihydroxy-6,11-dioxonaphthacenecarboxylate; Figure 1), and **5** (methyl 2-ethyl-5,7,-12-trihydroxy-4-[(2-hydroxyethyl)amino]-6,11-dioxonaphthacenecarboxylate) are isolated products from biofermentation process of *Streptomyces* strains purchased from Lividans Ltd. (Turku, Finland). Structures of **2** and **3** have been verified by ^{1}H NMR and mass spectrometry.

Human Recombinant Integrin I Domains and αI Domain Binding Assay. cDNAs encoding α1I, α2I, and α11I domains were generated by PCR as described earlier.[21−23] The α10I domain cDNA was generated by RT-PCR from RNA isolated from KHOS-240 cells (human caucasian osteosarcoma). Details of the cloning methodologies were described earlier.[23] Recombinant αI domains as a GST fusion protein were expressed and purified as described before,[21,23] and they were used for collagen experiments as such. Site-directed mutation of the α2I domain (glutamate 318 was mutated to tryptophan) cDNA in a pGEX-2T vector was carried out using PCR according to Stratagene's QuikChange mutagenesis kit instructions. The αI domain assay is described in detail elsewhere.[21,23] Nonlinear regression and the sigmoidal dose−response equation were used for data fitting.

Cell Adhesion Assay. Chinese hamster ovary (CHO) cell clones expressing α2 integrin were used in the cell adhesion assay. Cells were suspended in serum-free medium containing 0.1 mg/mL cycloheximide (Sigma), and the compounds were preincubated with the cells prior to transfer to the wells. Cells (150 000/well) were allowed to attach to collagen type I coated wells (in the presence and absence of inhibitor compounds) for 2 h at +37 °C and after that nonadherent cells were removed. Fresh serum-free medium was added, and the living cells were detected using a cell viability kit (Oncogene), according to the manufacturer's protocol. Mutations were generated as described before.[22]

Protein Structures. The three-dimensional structures of the integrin α2I domain, both in the "closed" (PDB access code: 1aox[24]) and "open" (PDB access code: 1dzi[3]) conformations, were obtained from the Protein Data Bank.[25] Structural comparison of the crystal structures was made with Vertaa in Bodil.[26]

Ligands. The molecular libraries of Lividans Ltd. (Turku, Finland) were used in this study. These libraries contained both real molecules and hypothetical molecules that are expected to be possible to produce using the biofermentation process. Both real and hypothetical molecules were modeled and stored in a molecular database for computer-based screening with the program Sybyl 6.9

(Tripos, St. Louis, U.S.A.). Each ligand structure was energy minimized prior to docking to the receptor model using the MMFF94s force field implemented in Sybyl until the conjugate gradient of energy was less than 0.05 kcal/mol. Ligands were docked flexibly into the "closed" conformation of the integrin α2I domain with the program FlexX in Sybyl. Ligands were suggested for experimental testing if they filled the following structural criteria for binding: the docked ligand had to (1) coordinate to Mg^{++}, (2) form interactions with αC helix, especially with Tyr285, and (3) fill the ligand binding cavity as much as possible, in particular, it was expected that the hydrophobic residues Leu286 and Leu296 would be screened by the ligand from the solvent. Ligands that showed inhibitory activity were analyzed more carefully. The process was repeated until the computational and experimental results were in consensus. The resulting models were then used to conceptualize the interaction between the ligands and the "closed" conformation of the integrin α2I domain.

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