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Fluorescent Small Molecule Probe to Modulate and Explore $\alpha 2\beta 1$ Integrin Function

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Supporting Information

ABSTRACT: Collagen binding integrins are an important family of cell surface receptors that mediate bidirectionally signals between the interior of the cell and the extracellular matrix. The protein−protein interactions between cells and collagen are necessary for many physiological functions, but also promote diseases. For example, the interaction of $\alpha 2\beta 1$ integrin and collagen has been shown to have an important role in thrombus formation and cancer spread. The fact that the discovery of small molecules that can block such protein−protein interactions is highly challenging has significantly hindered the discovery of pharmaceutical agents to treat these diseases. Here, we present a rationally designed novel fluorescent molecule that can be synthesized in just a few minutes from commercially available starting materials. This molecule blocks the protein−protein interaction between $\alpha 2\beta 1$ integrin and collagen, and due to its fluorescent properties, it can be employed in wide variety of biological applications.

Integrins are heterodimeric cell-surface proteins that anchor cells to their surrounding and mediate bidirectional signaling between the cytoplasm and the extracellular matrix or other cells.[1−3] From the currently known 24 integrin heterodimers, only four recognize collagens, $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$, and $\alpha 11\beta 1$. In contrast to other $\beta 1$-associated integrins, the α-subunit of these collagen receptor integrins contains an additional ∼200 amino acids-long inserted domain (αI domain; also known as A-domain) where collagens bind.[4,5] This αI domain masks the RGD-peptide binding site at the βI domain that has been widely used in small molecular discovery.[6] The available crystal structures of $\alpha 2\beta 1$ integrin I domain with (α2I; PDB-code: 1dzi;[7]) and without (Figure 1a, PDB-code: 1AOX[8]) bound collagen mimetic peptide reveal that collagen binds into a large surface area of the α2I metal ion-dependent adhesion site (MIDAS) (Figure 1a, orange surface), and changes drastically the conformation of α2I MIDAS (Figure 1a, red indicates the surface area drastically changed upon collagen binding). This conformational change likely controls the outside-in signaling.

The exact role of integrin-collagen interaction in harmful pathological conditions, such as the formation of blood clots and tumor metastasis, has been widely speculated.[9,10] Although various type of ligands have been developed, including (1) peptides derived from the snake venom of the Brazilian viper *Bothrops jararaca*,[11,12] (2) allosteric inhibitors,[13−16] and (3) nonallosteric inhibitors,[17,18] the development of $\alpha 2\beta 1$ integrin inhibitors toward drugs is hindered by the lack of a reliable, cost-effective, high-throughput screening method. Furthermore, there is an urgent need for tool molecules that could be used in reliable *in vitro* and *in vivo* experiments. This is especially important because in the cell-based assay the binding molecule is easily disengaged from the MIDAS by binding collagen. Accordingly,

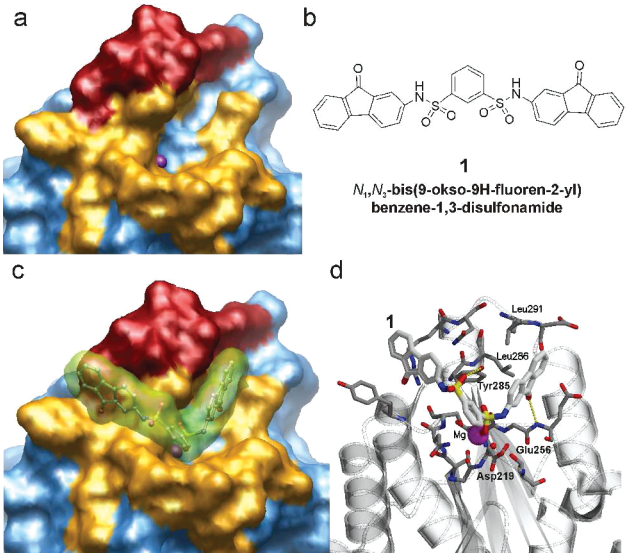

Figure 1. Binding of **1** to α2I domain. (a) Target protein PDB: 1AOX; (b) **1**, N_1,N_3-bis(9-oxo-9H-fluoren-2-yl)benzene-1,3-disulfonamide; (c and d) **1** docked into the collagen binding site of the α2I domain of the $\alpha 2\beta 1$ integrin. The hydrogen bonds are shown with orange dotted lines, hydrogen atoms are omitted for clarity. In (c), the surface of the α2I domain is shown, and in (d), the interactions between **1** and α2I domain are shown in detail. In (a) and (c), collagen mimetic peptide interaction site (orange surface), and the area that is changed during the collagen mimetic peptide binding process (red surface), are highlighted.

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the identification of inhibitors in high-throughput screens is atypical.

Here, we have employed a protein structure-based molecular discovery to develop a small molecular tool compound that can be employed in *in vitro* measurements, for example, in high-throughput screening and imaging as well as to understand the collagen binding in detail. Specifically, we sought competitive molecules for collagen binding into the α2I domain that have environment sensitive fluorescent properties. Similarly to most protein−protein interaction sites, the α2I domain surface is also relatively flat. Accordingly, the addition of a fluorescent label to known α2I ligands was not an applicable solution since the surrounding solvent can easily disengage the ligand if it contains parts that do not participate in the binding. The developed ligands are expected to block the collagen binding by hindering the conformational change of the I domain by interacting with (a) Mg^{2+} at MIDAS, (b) leucines 286 and 281 located at area where conformation of the I domain is changed most drastically (Figure 1a, red area), (c) main chain NH group of Glu256, (d) main chain oxygen of Asp219 (either direct or water mediated hydrogen bond), and (e) preferably with the side chain OH of Tyr285.[19] *De novo* modeling, based on the pharmacophore model, suggested that many widely used fluorophores, such as dansyl, coumarin, or anthracene derivatives, are not able to form interactions defined in the pharmacophore model, and additionally, the shape of these ligands is not complementary to the MIDAS of α2I domain. These observations were confirmed with synthesized dansyl derivatives that all were inactive (Supplementary Figure S1). In contrast, *de novo* modeling suggested that two fluorophores, fluorene and fluorenone, can be utilized in compounds that fulfill the set pharmacophore for α2I binding. All set requirements, that is, reasonable binding affinity, ability to inhibit $\alpha2\beta1$ integrin-collagen interaction, and appropriate fluorescent properties, are present in **CBL027** (**1**, N_1,N_3-bis(9-oxo-9H-fluoren-2-yl)benzene-1,3-disulfonamide; Figure 1b). Additionally, one aim during the ligand design was to keep the synthesis as simple as possible to facilitate the availability of the tool compound. The docking simulations predict that **1** can form both metal coordination to Mg^{2+} at the MIDAS, and hydrogen bonds with Asp219, Tyr285, and Glu256. Additionally, there are many favorable hydrophobic effects with the amino acids of the α2I that can hinder the collagen-induced conformational change (Figure 1c; red surface indicates the area that is substantially changed upon collagen binding). Moreover, the shape complementarity with the binding site is excellent, including the usage of a minor groove area at the surface (Figure 1c,d).

Compound **1** was prepared by single-step synthesis from commercially available starting materials 1,3-benzenedisulfonyl chloride and 2-amino-9-fluorenone using modified workup of previously reported procedures.[20] Compound **1** can be synthesized in minutes by using microwaves or by using overnight synthesis with conventional approach. The developed workup gave high purity product in moderate yield using simple recrystallization from water−ethanol mixture (see Supporting Information for more information about synthesis and characterization data, and structural figures; Figure S2 shows crystal structures of two polymorphs; crystallographic data is deposited at CSD). The fluorescent spectrum of **1** showed an emission maxima around 575 nm (Figure 2) when the excitation wavelength was set to 345 nm.

Many well-known fluorescent dyes, such as PRODAN[21] and 1,8-ANS,[22] indicate that the fluorescence of a molecule can alter when ligand binds to the protein surface and moves from an aqueous to a hydrophobic environment. Similar results have been reported with fluorenone when it forms complexes with cyclodextrins.[23] To investigate the behavior of **1** with proteins, the fluorescence of **1** was monitored when recombinant human α2I domain was added. The fluorescent intensity of **1** increased drastically when the α2I domain was added (Figure 2). To verify that the change in the fluorescence is caused by the ability of **1** to bind into α2I, and more specifically to its MIDAS region, the fluorescence of **1** was measured with three other compositions: (1) α2I domain with deleted helix αC that forms part of the binding site (Figure 1a, red);[24] (2) with the wild-type α2I domain in the absence of Mg^{2+} ion; and (3) with denatured protein. In contrast to α2I domain solution, the fluorescent intensity was not altered for any of these compositions (Figure S3). Consequently, the increase of fluorescence of **1** results from binding into MIDAS of the recombinant α2I domain.

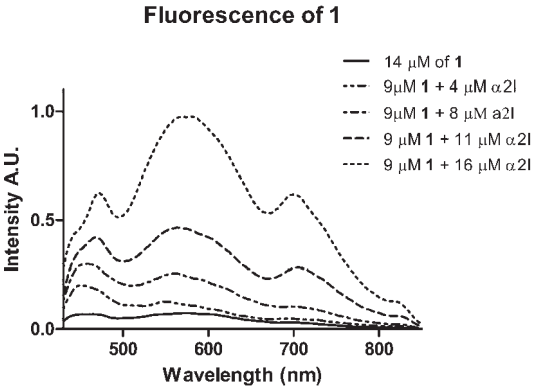

Figure 2. Corrected fluorescence emission spectra of **1** in a phosphate buffer. α2I domain was added to PBS (pH 7.4) containing 9 μM of **1** and 1 mM of MgCl; fluorescence (λ_{ex} = 345 nm) was recorded 4 min after adding the protein. One was first dissolved in DMSO and added to PBS. DMSO concentration was <2% in all samples. Temperature was 22−23 °C in all measurements. Emission values are normalized: (1) 14 μM of **1**; (2) 9 μM **1** + 4 μM α2I domain; (3) 9 μM **1** + 8 μM α2I domain; (4) 9 μM **1** + 11 μM α2I domain; (5) 9 μM **1** + 16 μM α2I domain.

In cell adhesion assay, compound **1** inhibited the adhesion of $\alpha2\beta1$ integrin overexpressing Chinese hamster ovary (CHO) cells to rat-tail collagen I with EC_{50} of 13 μM and maximal inhibition of 84% (Figure 3a). The cytotoxicity assay verified that the inhibitory effect of **1** is not caused by toxicity (cytotoxicity measured up to 200 μM). To confirm the binding site also in the full-length $\alpha2\beta1$ integrin, the inhibitor activity of **1** was tested with single-point Y285F mutation located at the MIDAS of α2I domain. The docked conformation of **1** forms a hydrogen bond into Y285, and accordingly, this Y285F mutation would lack this interaction (Figure 1c,d). As predicted, **1** does not inhibit collagen adhesion to CHO-α2Y285F cells at 15 μM concentration (EC_{50} 13 μM), which was determined with CHO-α2WT cells (Figure 3b). However, when the concentration of **1** is increased to 50 μM, it can also inhibit collagen adhesion in mutated cells (Figure 3b), supporting the premise that the binding site for **1** is at the MIDAS of α2I. **1** shows inhibitor activity also to $\alpha1\beta1$-collagen type IV adhesion (EC_{50} 17 μM; Figure S4), thus, it might also be possible to use it in a similar manner with $\alpha1\beta1$ integrin associated studies.

The fluorescent property of **1** upon binding to α2I domain was utilized in flow cytometry: Cells overexpressing $\alpha2\beta1$ integrin were labeled with **1** and detected in fluorescence-activated cell sorting (FACS) analysis (Figure 3c). Most suitable wavelengths

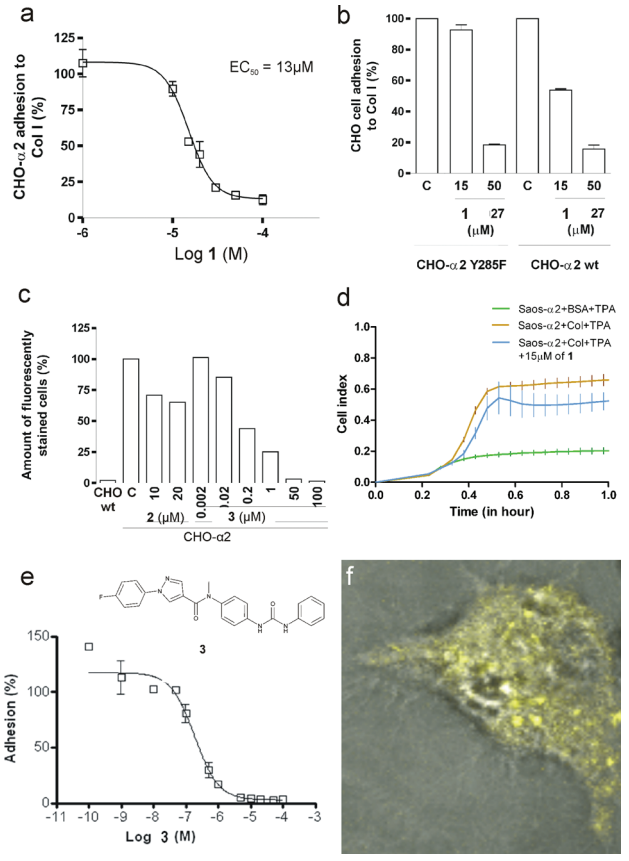

Figure 3. Results of **1** and **3** in cellular experiments: (a) **1** inhibits dose dependently the CHO-α2 cell adhesion to collagen I. Cells (150 000/well) were allowed to attach on rat-tail collagen I for 2 h at 37 °C. The number of adherent cells was measured with WST-1 (Roche Applied Science) according to the manufacturer's protocol. (b) **1** function is dependent on Y285 in α2I domain. CHO-α2 wild-type (wt) or CHO-α2 Y285F cells were allowed to attach on rat-tail collagen I for 2 h or 20 min at 37 °C, respectively. The number of adherent cells was measured with WST-1 according to the manufacturer's protocol. C indicates the control cells. (c) FACS results of **1** competed with **2** and **3**. CHO-α2 or CHO wild-type (wt) cells (106 cells/mL) were incubated with indicated concentrations of **2** or **3** for 30 min. After that, 50 μM fluorescing compound **1** was added and the incubation was continued for 1 h. The fluorescently labeled cells were detected with LSR II. C indicates CHO-α2 cell control. (d) xCELLigence real-time cell analyzer (Roche, Switzerland) was used to measure Saos-α2 cell adhesion to Collagen I (Col). Bovine serum albumin (BSA) served as a background control. Cells were pretreated with 100 nM TPA for 10 min at room temperature. Cell adhesion was followed for 1 h (37 °C, 5% CO_2) in the presence of 15 μM of **1**. The results indicate that **1** has only a minor effect on the adhesion of the inside-out activated Saos-α2 cells to collagen I. (e) **3** inhibits efficiently, EC_{50}: 190 nM, the CHO-α2 cell adhesion to collagen I. Measurements were done as for **1** in (a). (f) Confocal microscope image of CHO cell overexpressing $\alpha2\beta1$ integrin labeled with **1**. The wavelengths to detect **1** in the microscope were 405 or 458 nm for excitation and wavelengths over 560 nm for emission.

for detecting **1** in FACS measurements were determined at 430 nm for excitation and 540 nm for emission. FACS results with CHO-wt cells show that **1** is specific for collagen receptors since CHO-wt cells, which express other β1 integrins, were not detected after treatment with **1**.

The binding of **1** to inside-out activated integrin was studied using a phorbol ester tetradecanoylphorbol acetate (TPA) and xCelligence technology (Roche Applied Science). TPA is known to induce clustering of $\alpha2\beta1$ integrin and it has also supposed to change the conformation of α2I domain from closed to open in wild-type α2 integrin.[25] xCelligence measures changes in impedance at the bottom of a cell culture well caused by cell attachment and spreading. This method allows exploring the effect of **1** on cell adhesion in a time-dependent manner. We used Saos cells that were stably transfected with human integrin α2 subunit. The experiments were carried out in the presence of 15 μM **1** in serum-free DMEM. The cells were activated with TPA, and subsequently, cell adhesion was followed for 1 h in the presence of 15 μM **1**. As a result, **1** had only a minor effect on the adhesion of the inside-out activated Saos-α2 cells to collagen I (Figure 3d and Figure S5, see Supporting Information for detailed information). This indicates that TPA effects the α2I domain conformation, and furthermore, that **1** binds to the closed conformation of the α2I domain.

To verify the suitability of FACS in screening, we competed the previously developed α2I domain ligand L3008 (**2**, methyl 2-ethyl-4,5,7,12-tetrahydroxy-6,11-dioxonaphthacenecarboxylate,[17] see Supporting Information for detailed information) with **1**. Cells were first incubated with **2** and followed by treatment with **1**. Flow cytometry experiment shows concentration depended reduction in the amount of fluorescent cells when the concentration of **2** was increased (Figure 3c). Consequently, results indicate that the binding of **1** and **2** is competitive. **1** and **2** have very similar binding efficiencies. However, in contrast to **1**, **2** is shown to be cytotoxic at concentration above 50 μM; hence, it cannot be reliably used in cell assays.[17] The developed flow cytometry method was then employed by screening a series of compounds around the previously identified α2I inhibitors.[17−19] This screen identified a highly potent inhibitor **3** (1-(4-fluorophenyl)-1H-pyrazole-4-sulfonic acid *N*-methyl-*N*-[4-(3-phenylureido)-phenyl]amide, see Supporting Information for detailed information) for collagen adhesion to CHO-$\alpha2\beta1$ cells. Compound **3** is not cytotoxic (up to 200 μM) and in cell adhesion assay inhibits collagen adhesion to CHO-$\alpha2\beta1$ cells in nanomolar concentration (EC_{50}: 190 nM) with maximal inhibition of 97% (Figure 3e). Since both **2** and **3** can block dose dependently the binding of **1** to the $\alpha2\beta1$ integrin surface of CHO-α2 cells, **1** can be most likely employed to map the binding site of integrin ligands. Furthermore, **1** can be used to develop a new high-throughput screening method for the discovery of novel $\alpha2\beta1$ integrin ligands.

The fluorescence property of **1** upon binding to CHO-α2 cells was also tested in fluorescence microscopy (Zeiss LSM510 Meta). Cells were labeled with **1** and detected in a confocal microscope (Figure 3f). The wavelengths to detect **1** in the microscope were 405 or 458 nm for excitation and wavelengths over 560 nm for emission. These results indicate that **1** can be used as a tool to investigate the expression and function of α2 integrin with fluorescent microscopy methods.

In conclusion, we have developed a powerful tool molecule, **1**, to explore $\alpha2\beta1$ integrin function at both the molecular and cellular levels. This molecule competitively blocks the collagen binding to αI domain of $\alpha2\beta1$ integrin, and it can be used as a fluorescent marker, instead of expensive antigens, for cells expressing $\alpha2\beta1$ integrin, for example, blood platelets that do not express the other collagen binding integrins. **1** binds also to $\alpha1\beta1$, and accordingly, it can be used as a general tool to study collagen binding integrins. However, it must be kept in mind that

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1 cannot bind into $\alpha2\beta1$ integrin, for example, in the absence of magnesium or when drastic mutations are introduced (Figure S3). The FACS results indicate that the fluorescence property of **1** can be employed to develop a high-throughput screening method for the identification of $\alpha2\beta1$ integrin function modulators. In addition, **1** can be utilized in microscopic studies to understand in detail the role of $\alpha2\beta1$ integrin in, for example, both thrombus formation and spreading of malignant cells, two pathological conditions that are widely connected to the $\alpha2\beta1$ integrin expression and function. Therefore, it is likely that this compound will stimulate the discovery of $\alpha2\beta1$ integrin inhibitors and their usage as therapeutic agents.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details, characterization data, and cif files. This material is available free of charge via the Internet at http://pubs.acs.org.

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