Selectivity of the collagen-binding integrin inhibitors, TC-I-15 and obtustatin

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ARTICLE INFO

Editor: Dr. Lawrence Lash

Keywords:
Integrin
Collagen peptides
Obtustatin
TC-I-15
Cell adhesion
C2C12

ABSTRACT

Integrins are a family of 24 adhesion receptors which are both widely-expressed and important in many physiological cellular processes, from embryonic development to cancer metastasis. Hence, integrin inhibitors are valuable research tools which may have promising therapeutic uses. Here, we focus on the four collagen-binding integrins α1β1, α2β1, α10β1 and α11β1. TC-I-15 is a small molecule inhibitor of α2β1 that inhibits platelet adhesion to collagen and thrombus deposition, and obtustatin is an α1β1-specific disintegrin that inhibits angiogenesis. Both inhibitors were applied in cellular adhesion studies, using synthetic collagen peptide coatings with selective affinity for the different collagen-binding integrins and testing the adhesion of C2C12 cells transfected with each. Obtustatin was found to be specific for α1β1, as described, whereas TC-I-15 is shown to be non-specific, since it inhibits both α1β1 and α11β1 as well as α2β1. TC-I-15 was 100-fold more potent against α2β1 binding to a lower-affinity collagen peptide, suggestive of a competitive mechanism. These results caution against the use of integrin inhibitors in a therapeutic or research setting without testing for cross-reactivity.

1. Introduction

Integrins are a family of glycoprotein transmembrane cell adhesion receptors that exist as α/β heterodimers. In humans, there are 18 α-subunits and 8 β-subunits that combine to form 24 different integrins (Arnaout et al., 2005; Barczyk et al., 2010; Hynes, 2002). Integrin expression is widespread but cell type-dependent, and most integrins bind a selection of extracellular matrix (ECM) components or cell-surface ligands. It is thought that cells express an excess of β-subunits and the expression of α-subunits determines surface receptor expression (Santala and Heino, 1991). They play essential roles in embryonic development, cell migration, proliferation and angiogenesis, and have been implicated in tumorigenesis and inflammation (Santala and Heino, 1991; Arnaout, 1996; van der Flier et al., 2010; San Antonio et al., 2009; Pozzi et al., 1998; Zhang et al., 2008; Senger et al., 2002; da Silva et al., 2010; Sottini et al., 2013; Alique et al., 2014). Their primary function is to facilitate adhesion of cells to each other and to the ECM, but also to take part in matrix assembly (Musiime et al., 2021). Integrin-mediated signalling is essential for cell survival and many cell responses to growth factors are dependent on cell adhesion to a substrate via integrins (Barczyk et al., 2010; Meredith Jr. and Schwartz, 1997; Schwartz and Assoian, 2001; Byzova et al., 2000). As a consequence, many cell types must adhere to the matrix through integrins to survive (Schwartz and Assoian, 2001; Assoian, 1997).

Both the α and β subunits have a large extracellular N-terminal ‘head’ domain, a transmembrane domain and a small cytoplasmic C-terminal ‘tail’ (Arnaout et al., 2005; Barczyk et al., 2010; Hynes, 2002; Takada et al., 2007). By linking the ECM to the cytoskeleton, integrins mediate signal transduction from the environment, activating downstream signalling pathways such as focal adhesion kinases, talin and Src family kinases (Huveneers and Danen, 2009; Tadokoro et al., 2003; Calderwood et al., 1999). They form bi-directional signalling hubs that coordinate signals from several pathways (Arnaout et al., 2005; Giannotti and Ruoslabi, 1999) and form clusters with co-receptors and other integrins to amplify and modulate signals (Wolf et al., 2012).

The four collagen-binding integrins, α1β1, α2β1, α10β1 and α11β1 all adhere to the GFOGER motif found primarily in collagens I and II, and to GLOGEN that is unique to collagen III. α2β1 and α11β1 adhere more strongly to GFOGER while α1β1 and α10β1 adhere more strongly to GLOGEN. Triple-helical peptides (THPs) containing these motifs have

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https://doi.org/10.1016/j.taap.2021.115669
Received 13 May 2021; Received in revised form 29 July 2021; Accepted 2 August 2021
Available online 5 August 2021
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been synthesised and are used throughout this study (Emsley et al., 2000; Farndale, 2019; Knight et al., 2000; Zhang et al., 2003). Integrins α1β1 and α2β1 are widely expressed, whereas α10β1 is expressed primarily in chondrocytes (Barczyk et al., 2016; Takada et al., 2007) and α11β1 is expressed in fibroblasts, subsets of mesenchymal stem cells and subsets of cancer-associated fibroblasts (Zeltz and Gullberg, 2016; Popova et al., 2007; Popov et al., 2011; Shen et al., 2019; Zeltz et al., 2019; Zeltz et al., 2020). The range of pathways involving integrins highlights their importance in tissue function and homeostasis across a variety of organs. For example, whole organism ablation of β1 integrin in mice leads to embryonic fatal phenotype before E5.5 (Fassler and Meyer, 1995). Similar ablation of α1 or α2 subunits has a much less severe effect, with α1-null mice producing viable offspring with minor defects in collagen synthesis and angiogenesis (Gardner et al., 1996; Pozzi et al., 2000), whilst α2-null mice show delayed platelet aggregation (Chen et al., 2002). Both α1β1 and α2β1 enhance cancer cell migration and metastasis (Primac et al., 2019), for example, by upregulating matrix metalloproteinase synthesis via MAPK signalling (Ibaragi et al., 2011). Integrin α2β1 also promotes prostate cancer metastasis to the skeleton, resulting in a poor prognosis for patients (Sottink et al., 2013). Unlike α1β1 and α2β1, which in tumours are expressed in both neoplastic and vascular cells, α11β1 is restricted to cancer-associated fibroblasts, and in a breast cancer model, restricts both tumour growth and metastasis (Primac et al., 2019). Understanding the inhibition of collagen-binding integrins is central to therapeutic targeting of integrins in these and other pathologies.

Nine of the integrins, including α1, α2, α10 and α11, contain an α-domain, an insertion of about 200 amino acids in the β-propeller structure of the α subunit head. The β subunits contain a similar β-domain, and together, these I-domains regulate the activation status of the integrin (Arnauot et al., 2005; Shimaoka et al., 2002). The α-domain determines ligand specificity despite their high homology; the rest of the subunit is more variable (Luo et al., 2007). The I-domain contains a metal-ion-dependent adhesion motif from collagen or THPs (Emsley et al., 2000; Dickeson and Santoro, 2013). Unlike α1 and α2, which in tumours are expressed in both neoplastic and vascular cells, α11β1 is restricted to cancer-associated fibroblasts, and in a breast cancer model, restricts both tumour growth and metastasis (Primac et al., 2019). The I-domain MIDAS (Miller et al., 2009) is considered to inhibit small molecule inhibitor, TC-I-15 (compound 15 in (Miller et al., 2009)). E318 is located at the top of helix 7, forming a salt bridge with R288 in the C-helix which must be broken to permit activation (Emsley et al., 2000). Thus, in E318A, helix 7 is decoupled from the upper surface of the α-domain which can reorganise independently of the β-domain, and so E318A cannot be stabilised in the inactive conformation by TC-I-15.

Disintegrins are a family of integrin inhibitors derived from snake venoms (Arruda Macedo et al., 2015; Marckinkiewicz et al., 2003; Daidone et al., 2013). They are small cysteine-rich polypeptides, divided into several sub-groups based on their size or specificity. Disintegrins contain an integrin binding loop that, generally, competes directly with the natural ligand at its β-subunit binding site, and to fulfil this function, the integrin binding loop contains an RGD motif (Arruda Macedo et al., 2015). Similar motifs include MLD, VGD, KGD, and WGD, with each motif conferring a degree of integrin selectivity (see Table 1). Obtustatin, a KTS-disintegrin of just 41 residues, differs in its integrin-binding loop which contains WKTSLTSHY (Marckinkiewicz et al., 2003; Daidone et al., 2013), where the threonine residue (T22) is essential for α1β1 binding and the adjacent leucine (L24) contributes to high affinity (Kisel et al., 2004). Two further KTS-disintegrins, vipersistant and leb-estatin, and an RTS-containing disintegrin, jerdostatin, are also potent inhibitors of α1β1 (Kisel et al., 2004), although where these disintegrins bind to α1β1 is not known. Obtustatin inhibits cellular, membrane-bound α1β1 and the isolated full length α1β1 but has no effect on re-combinant α-domains (Marckinkiewicz et al., 2003). The specificity of obtustatin, together with the absence of an acidic residue in its integrin-binding loop, suggests that obtustatin must interact with the α1 subunit, most likely as well as with the β1 subunit, close to the interface between the two. Obtustatin has been used to inhibit angiogenesis in vitro and in vivo in chichoroaallanotic membrane assays (Marckinkiewicz et al., 2003). Obtustatin also reduced tumour development by 50% in the mouse Lewis lung carcinoma model, and blocked melanoma growth in mice (Marckinkiewicz et al., 2003; Brown et al., 2008).

Neither of these two inhibitors, TC-I-15 and Obtustatin, has been tested on the more recently-characterised integrins, α10β1 or α11β1. Here, we tested their cross-reactivity with other collagen-binding integrins using recombinant integrin α-domains, HT1080 cells that express only α2β1 and C2C12 cells that have been stably transfected to express only α1β1, α2β1, α10β1 or α11β1. A previously-characterised inhibitor of α2β1, monoclonal antibody 6F1, was also tested for comparison. The commercially-available small molecule α2β1 inhibitor, TC-I-15, was found to be non-specific as it also exerted an inhibitory effect on α1β1 and, at much higher concentrations, α11β1. Further, TC-I-15 was found to have no effect on α10β1 or purified α3β1. However, Obtustatin was found to inhibit only α1β1 of the four collagen-binding integrins. This highlights the importance of rigorously testing inhibitors for cross-reactivity before they can be used specifically.

### 2. Materials and methods

Integrin α-domains were expressed as described (Siljander et al., 2004; Hamaia et al., 2012). TC-I-15 (4527/10), Obtustatin (4664/100 U) and α3β1 (2840-A3-050) were purchased from R&D technologies. Placental laminin-S11 was purchased from Sigma L6274 (Wondimu et al., 2006). TC-I-15 was dissolved in NaOH (typically 220 μM) to obtain a final molar ratio of 1:1.1 (TC-I-15:NaOH). NaOH alone was used as a vehicle control for TC-I-15 whereas obtustatin and 6F1 were used as a vehicle control for obtained and TC-I-15.
suspended in PBS. 6F1 was the generous gift of Dr. B. Coller, NY, USA.

2.1. Cell culture

C2C12s and HT1080s were grown in Lifetech DMEM/10% FBS and 1% penicillin/streptomycin. All cells were maintained under sterile conditions at 37 °C, 5% CO2. Cells were passaged using Trypsin/EDTA at 37 °C for 5 min and DMEM/10% FBS to quench the trypsin. C2C12 cells were transfected as described previously (Tiger et al., 2001). The C2C12-a10 clone was kindly made available by Dr. Evi Lundgren-Åkerlund, Xintela AB, Sweden.

2.2. Cellular static adhesion assays

Immunol 2HB 96-well plates were coated with 100 µl per well of peptides at 10 µg/ml in 0.01 M acetic acid overnight at 4 °C. After 3 × 200 µl/well PBS washes, plates were blocked with 200 µl/well filtered 3% BSA in PBS at RT for 1 h. Plates were washed, and 20,000 cells/well were added at room temperature (RT) for 1 h in serum-free media with 5 mM MgCl2 and varying concentrations of inhibitor. Unbound cells were washed away with 3 × washes of 200 µl/well PBS. Adherent cells were lysed with 50 µl per well of 2% Triton X-100 in water for 1 h at RT. Cell number was quantified using 50 µl per well of the Roche cytotoxicity LDH kit. The substrate and substrate solutions of the kit were mixed at a ratio of 1:45 and 50 µl was added to the wells to detect LDH in the cell lysate. A500 was read in a SpectraMax 190 microplate reader (Molecular Devices). Each condition was performed in triplicate, with at least three independent repeats.

2.3. Protein static adhesion assays

Immunol 2HB 96-well plates were coated with 100 µl per well of peptides at 10 µg/ml in 0.01 M acetic acid with washing buffer (1 mg/ml BSA in TBS), plates were blocked with 200 µl per well of filtered 3% BSA in TBS for 1 h at RT. ad-domains were added at 10 µg/ml in washing buffer for 1 h at RT with 5 mM MgCl2 and various concentrations of inhibitor as stated. After 3 × 200 µl/well washes with washing buffer, anti-GST detection antibody (GE Healthcare HRP conjugated anti-GST GERPNI236) were added for 1 h. Plates were washed 4 × with 200 µl per well washing buffer and bound protein was quantified using the Pierce™ TMB Substrate Kit. The colorimetric reaction was stopped with an equal volume of 1 M H2SO4 and A450 was read as above. Each condition was performed in triplicate and each experiment was repeated at least three times using different preparations of proteins.

2.4. Collagen peptides

Peptides containing the sequences GFOGER or GLOGEN (single amino acid nomenclature where O is hydroxyproline) were synthesised as C-terminal amides, and assembled as triple-helical homotrimers, as described previously (Knight et al., 2000; Raynal et al., 2006). Peptides contain a binding motif (such as GFOGER) flanked on either side by five GPP repeats and a GPC triplet at both the N- and C-termini. Peptides were synthesised using solid phase Fmoc peptide chemistry on a CEM Molecular Devices). Each condition was performed in triplicate, with at least three independent repeats. Means ± SD are shown in all data sets. Inhibition curves were analysed using nonlinear regression comparing each data set with either a horizontal line model (null hypothesis, no inhibition) or three-parameter dose-response curve (inhibition occurs). Three-parameter curves were constrained with lower value <0.1, reflecting inhibition of adhesion to baseline GPP10 values, and IC50 > 0.

3. Results

3.1. Integrin α1-domain adhesion to collagen peptides is unaffected by TC-I-15 and Obtustatin

To confirm that these inhibitors have no effect on isolated α-domains, GST-tagged α-domains were tested in static adhesion assays where plates had been coated with either GLOGEN (for the α1-l-domain) or GFOGER (for the α2-l-domain) in the presence or absence of the inhibitors obtustatin, 6F1 and TC-I-15. Previous work in this laboratory established that GFOGER is a strong ligand and GLOGEN a moderate ligand for α2β1 whereas GLOGEN is a strong ligand and GFOGER a moderate ligand for α1β1 (Farndale, 2019; Knight et al., 2000; Farndale et al., 2008). The α2β1 inhibitory antibody, 6F1, was also included for comparison (Coller et al., 1989). Fig. 1A and B show levels of adhesion after inhibition. Antibody 6F1 was a potent inhibitor of the α2-l-domain adhesion to GFOGER (control mean = 2.51 ± 0.3; 6F1 mean = 0.29 ± 0.056, p < 0.0001) but not the α1-l-domain adhesion to GLOGEN, confirming its specificity for α2β1. This confirms that, whereas 6F1 directly targets the free α1-domain, obtustatin and TC-I-15 are not able to do so, in agreement with the literature.

3.2. In C2C12 cells, TC-I-15 inhibits adhesion of α1β1, α2β1 and α11β1, but not α10β1, to collagen peptides

Next, to test the effects of these inhibitors on the full-length integrin receptors, C2C12 cells that have been stably transfected to express one of the four integrins were used in static adhesion assays. Plates were coated with GFOGER, GLOGEN or GPP10 (as the negative control) and inhibition dose curves for TC-I-15 and Obtustatin were carried out. Fig. 2 shows the dose curves for TC-I-15-mediated inhibition of the adhesion of C2C12 cells expressing α1β1, α2β1, α10β1 or α11β1 (Fig. 2A-2D respectively). Non-linear regression was used to analyse the curves, as described in Materials and Methods. Firstly, TC-I-15 inhibited α2β1-expressing C2C12 cell adhesion in dose-dependent manner, as anticipated (for GFOGER, IC50 = 26.8 µM, R2 = 0.9066, P < 0.0001 and for GLOGEN IC50 = 0.4 µM, R2 = 0.9943, P < 0.001), but it also inhibited α1β1 (for GFOGER, IC50 = 23.6 µM, R2 = 0.98, P < 0.001 and for GLOGEN IC50 = 24.4 µM, R2 = 0.94, P < 0.001). At higher concentrations, α1β1 was also inhibited (for GFOGER, IC50 = 3177 µM, R2 = 0.80, P < 0.001 and for GLOGEN IC50 = 177 µM, R2 = 0.70, P < 0.001). For TC-I-15-mediated inhibition of α10β1, and all the control conditions, the preferred statistical model was a horizontal line, i.e., no inhibition was seen. Secondly, the degree of inhibition was peptide-dependent for α2β1 and α11β1, but not for α1β1. For α2β1, complete inhibition of adhesion was seen at very low concentrations of TC-I-15 for the lower-affinity peptide, GLOGEN, but a much higher concentration of TC-I-15 was needed to achieve the same effect for adhesion to GFOGER. A similar substrate-dependence was seen with α11β1. In contrast, for α10β1 the potency was the same for both peptides tested and the IC50 values are very similar.
3.3. In C2C12 cells, obtustatin inhibits α1/β1 only

The inhibition dose curve assays were repeated with obtustatin (Fig. 3) and non-linear regression was used to analyse the curves as above. Here, obtustatin caused potent inhibition of the adhesion of α1/β1 (Fig. 3A) to GFOGER (IC50 = 0.45 μM, R2 = 0.8920, P < 0.0001) and GLOGEN (IC50 = 0.96 μM, R2 = 0.8496, P < 0.0001). The difference between inhibition on the two peptides was not significant. For the other transfected cells (Fig. 3B-D), no inhibition was observed. This suggests obtustatin is an efficient and specific inhibitor of full length α1β1.

3.4. Adhesion of HT1080s is inhibited by TC-I-15 but not obtustatin

To confirm these findings in a second cell type, inhibition experiments were repeated by measuring the adhesion of HT1080 cells, which express α2β1 but not α1β1 (Ruggerio et al., 1996), to GFOGER (Fig. 4). Obtustatin had no effect on HT1080 adhesion (Fig. 4A). Notably, TC-I-15 inhibited the adhesion of HT1080 cells to GFOGER, P < 0.0001 compared to vehicle control, and at lower concentrations than adhesion of C2C12-α2 cells, with IC50 4.53 μM and 26.77 μM, respectively (Fig. 4B). HT1080 is a human fibrosarcoma line, and so expresses both the human α- and β-subunits, whereas C2C12 cells are mouse myofibroblasts, stably transfected with the human α-subunit that dimerises with the mouse β-subunit. Possibly TC-I-15 may have lower affinity for the mouse β-subunit, or the human-mouse heterodimer may communicate imperfectly. HT1080 cells may express lower levels of α2β1, leading to lower avidity of binding to the peptide-coated surfaces. Any of these effects could explain the differences in IC50 values for TC-I-15 inhibition shown here.

3.5. Full-length recombinant integrin α3β1 is not affected by TC-I-15

Here, TC-I-15 has been shown to have broader specificity of inhibition of β1 integrins than reported to date. Since several other integrins contain the β1 subunit (the laminin-binding integrins, α3β1, α6β1, and α7β1, the RGD-binding integrins, α5β1 and α6β1, and the leukocyte integrins, α4β1 and α9β1), we considered that TC-I-15 might also inhibit other β1 integrins. As a representative example of such receptors, adhesion of full-length recombinant human α3β1 was tested with TC-I-15, using laminin 511 as the substrate (Fig. 5). In these conditions, TC-I-15 showed no effect on α3β1.

4. Discussion

Currently, TC-I-15 is available commercially as an inhibitor for α2β1. Our results show that TC-I-15 is not specific, but has a broader specificity including α1β1, α2β1 and, at a much higher concentration, α1β1. In contrast, TC-I-15 has, at best, slight effect on the adhesion of α10β1 to GFOGER or GLOGEN peptides. The inhibition is substrate-dependent for α2β1 and α11β1, in that TC-I-15 inhibits adhesion to the lower-affinity GLOGEN at a much lower concentration than applies to GFOGER. The difference in potency of TC-I-15 inhibition of adhesion to these two ligands suggests a competition, where inhibition of adhesion to the higher-affinity GFOGER requires a higher concentration of TC-I-15 than inhibition of binding to GLOGEN. This suggests a reciprocal relationship between GFOGER binding to the upper surface of the αI-domain and TC-I-15 recognition of its inhibitory site on the β-domain. In contrast, the lower affinity GLOGEN does not require such a high concentration of TC-I-15 to achieve the same effect. Interestingly, this difference in potency is not seen for α1β1, where both peptides are inhibited by similar concentrations of TC-I-15, presumably reflecting the similar affinity of α1β1 for GLOGEN.

Crystal structures provide clear insight into the movement of helix 7 and the C-helix in the α2 I-domain upon binding of GFOGER (Emsley et al., 2000), and the ligation of E336 at the foot of helix 7 by the β-subunit MIDAS (Carafoli et al., 2013). The location of TC-I-15 binding close to the α2-β1 interface is also well described (Miller et al., 2009), and its effect is understood as allosteric, stabilising the inactive conformation of the integrin. The apparent competition between GFOGER or GLOGEN and TC-I-15 is, however, consistent with this allosteric model. Competition occurs between α3β1 and TC-I-15 at the β1-domain MIDAS, while the role of the collagen peptide is to drive α helix 7 downwards closer to the β1 MIDAS. In activated platelets, the affinity of α2β1 for weaker ligands increases, whereas binding of the high

Fig. 1. Inhibition of binding of recombinant αI-domains to peptide coatings. Adhesion of α1, α1 I-domain to GLOGEN and α2 I-domain to GFOGER, was measured using anti-GST-HRP conjugated antibody as described in Materials and methods, and is shown as mean A450 ± SD. TC-I-15 (200 μM), Obtustatin (20 μM) or 6F1 (10 μg/ml) were used as indicated. “TC-I-15 ctrl” refers to the vehicle control, 220 μM NaOH. Each condition was performed in triplicate and repeated 3 times. **** denotes P < 0.0001.
affinity GFOGER is less dependent upon cellular stimulation (Siljander et al., 2004). Thus, in resting cells, GFOGER can provide sufficient binding energy to trigger the reorganisation of the α-subunit MIDAS, C-helix and helix 7 without inside-out integrin activation. This process must include the disruption of the E318 salt bridge, allowing helix 7 to translocate downwards towards the βI-domain MIDAS. A weaker ligand for α2β1, in this case GLOGEN, is unable to provide the same binding energy, and TC-I-15 is more readily able to inhibit α2β1 by blockade of the helix 7–βI MIDAS interaction. Hence, helix 7 couples ligand binding at the α-subunit MIDAS to competitive inhibition by TC-I-15 at the β-subunit MIDAS.

The question of selectivity of both TC-I-15 and obtustatin for the different collagen-binding integrins is more difficult to explain. We assume that both inhibitors must interact with both the βI-domain MIDAS and part of the α-subunit at the α–β interface. There is no crystal structure of an intact integrin that contains an αI-domain, and although this interface has been modelled (Miller et al., 2009), no authentic data exists and the relationship between the αI- and βI-domains is not clear. Inspection of the four αI-domain sequences shows several differences in helices 1, 6 and 7, the region of the α-subunit most likely to contribute to the footprint of the inhibitors upon these integrins. These may contribute to the lower affinity of TCI-15 for α10 and α11 and for the very high selectivity of obtustatin.

We show here, through the use of recombinant α-domains, that obtustatin does not compete directly with collagen at the αI-domain. Instead, obtustatin may interact with α1β1 in a similar way to other disintegrins, by binding to the βI-domain at or close to the α–β interface. The action of obtustatin could be analogous to that of TC-I-15, by stabilising both the inactive conformation of the βI-domain and blocking the reorganisation of helix 7 and the C-helix. Structural work is needed to clarify how the selectivity and potency of the active KTSL motif is achieved.

Given that TC-I-15 interacts primarily with the β1 subunit to stabilise the inactive conformation of α1β1 and α2β1, we considered whether TC-I-15 might also inhibit other β1 integrins that lack an αI-domain. The previous report (Miller et al., 2009) had shown no effect of TC-I-15 on the adhesion of platelets, which express α5β1 and α6β1, to the RGD-containing ligands, fibronectin and fibrinogen. Here, we verify this specificity by showing that TC-I-15 had no effect on binding of full-length purified α3β1 to the LDV-containing laminin-511, confirming that TC-I-15 is not a universal inhibitor of β1 integrins. This reinforces the concept that the TC-I-15 interaction involves both the β subunit and the αI-domain. However, the lack of effect on integrin α10β1, which also contains an αI-domain, suggests that the presence of both the β1 subunit and an αI-domain is not sufficient for inhibition to occur. The molecular detail of the interaction remains to be resolved.

In conclusion, it is imperative that integrin inhibitors are thoroughly tested for cross-reactivity before any therapeutic or research use.

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**Fig. 2.** Dose-dependent inhibition of integrin-mediated adhesion of C2C12 cells by TC-I-15. C2C12 cells stably expressing either A, α1β1, B, α2β1, C, α10β1 or D, α11β1 were tested for adhesion to GFOGER (blue) and GLOGEN (red) peptides as described in Materials and methods. NaOH (maximum concentration of 550 μM) was included as a vehicle control on GFOGER (green) and GLOGEN (orange). Adhesion to GPP10 in the absence of inhibitor or vehicle control is shown as the baseline (purple). Adhesion is shown as Mean A490 ± SD. Each condition was performed in triplicate and repeated three times. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Obtustatin, in these conditions, was a potent and specific inhibitor for $\alpha_1\beta_1$ adhesion to collagen peptides. The data presented here supports the use of obtustatin for the specific inhibition of $\alpha_1\beta_1$ in cell-based assays, in-vivo assays or potential therapeutic settings. However, because TC-I-15 inhibits $\alpha_1\beta_1$, $\alpha_2\beta_1$ and, at high concentration, $\alpha_{10}\beta_1$, it must be used with caution in cellular environments where more than one of these integrins is present. TC-I-15 would be a useful tool when inhibition of both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ is required, or where only one of these
receptors is present. For example, TC1-15 been proposed as a potential antithrombotic agent and has been used to block α2β1 on the surface of platelets to inhibit collagen-stimulated platelet aggregation (Miller et al., 2009). Platelets do not express α1β1 or α11β1 and so TC1-15 would work well here as a specific inhibitor of α2β1. However, in cell-based assays or in-vivo experiments, where more than one of the integrins α2β1, α1β1 and α11β1 are present, such as fibroblasts, endothelial cells or circulating cells such as leukocytes, the cross reactivity of TC1-15 should be considered.

Declaration of Competing Interest

RFW is Chief Scientific Officer, CambCol Laboratories, Ely, Cambs, UK.

Acknowledgments

This study was funded by grants from the British Heart Foundation: SP/15/7/31561 to RF; RG/15/4/31268 to RF, and FS/15/20/31335 to RF and EH.

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