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Relative Binding Affinities of Integrin Antagonists by Equilibrium Dialysis and Liquid Chromatography–Mass Spectrometry

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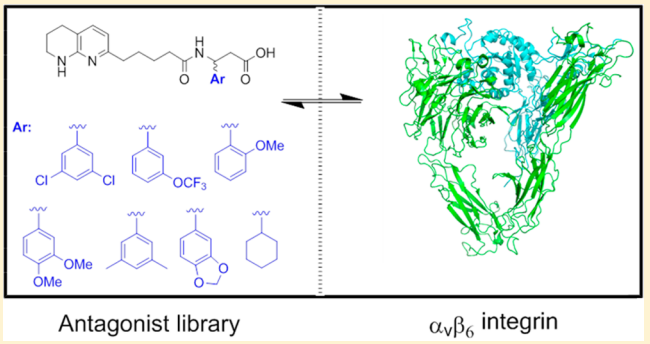
Supporting Information

ABSTRACT: The integrin $\alpha_v\beta_6$ is a potential target for treatment of idiopathic pulmonary fibrosis (IPF). Equilibrium dialysis (ED) was investigated for its ability to report ligand binding in an $\alpha_v\beta_6$ inhibitor screening assay. As a preliminary experiment, an established peptidomimetic inhibitor of the integrin was dialyzed against $\alpha_v\beta_6$, and the fraction bound (f_b) and percentage saturation determined by liquid chromatography–mass spectrometry (LC-MS) analysis. Quantitation of the inhibitor in the two chambers of the ED cartridge revealed an uneven distribution in the presence of $\alpha_v\beta_6$, corresponding to near saturation binding to the protein (93 ± 3%), while the control (without integrin) showed an equal partitioning of the inhibitor on either side of the dialysis membrane. A competitive ED assay with a 12 component mixture of antagonists was conducted, and the results compared with an established cell adhesion assay for quantifying $\alpha_v\beta_6$ inhibition of individual antagonists. Compounds clustered into three groupings: those with pIC_{50} values between ca. 5.0 and 5.5, which possessed ED f_b values indistinguishable from the controls, those with pIC_{50}s of 6.5 ± 0.2, which exhibited detectable integrin binding (f_b 13–25%) in the ED assay, and a single compound of pIC_{50} 7.2 possessing an f_b value of 38%. A good correlation between ED-derived f_b and pIC_{50} was observed despite the two assays utilizing quite different outputs. These results demonstrate that ED with LC-MS detection shows promise as a rapid $\alpha_v\beta_6$ integrin antagonist screening assay for mixtures of putative ligands.



KEYWORDS: *Idiopathic pulmonary fibrosis, $\alpha_v\beta_6$, integrin, equilibrium dialysis, liquid chromatography–mass spectrometry*

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive disease of unknown etiology.[1] It is characterized by scarring of the lung tissue, which leads to shortness of breath (dyspnea), a dry cough, inspiratory bibasilar crackles, and fatigue.[2] The prognosis for IPF sufferers is poor, with a 5-year mortality rate higher than that of many common cancers.[3] Recent studies estimate the prevalence of IPF among adults in the USA to be between 14.0 and 43.7 per 100,000, with the majority of cases in the over-50 age group.[4] Currently there is no effective pharmacological treatment for IPF, and lung transplantation remains the only recognized procedure. IPF, therefore, represents a disease in dire need of therapeutic intervention.

Although the cause of IPF is unknown, its pathobiology is closely linked to dysregulation of transforming growth factor-β1 (TGF-β1).[5] This cytokine is a key mediator of epithelial cell repair, promoting apoptosis and cell migration, which, if overactive, may result in the lung scarring seen in IPF sufferers. TGF-β1 is expressed as an inactive noncovalent complex with latency associated peptide (LAP).[6] Activation of the cytokine is promoted by proteases or by the action of the integrin family of signaling protein, for example $\alpha_v\beta_6$.[6] Integrins are heterodimeric membrane-associated proteins found on the surfaces of many cells. Crucially, the distribution of $\alpha_v\beta_6$ is restricted to epithelial cells,[6] and so specific inhibition of this integrin may provide a mechanism for effective treatment of IPF through targeted down-regulation of TGF-β1 activity in this cell type.

All members of the α_v subfamily of integrins activate TGF-β1 by binding to an RGD tripeptide motif on LAP. The resulting α_v·LAP·TGF-β1 complex is believed to mediate cytokine activation either by a protease-independent conformational change or by recruitment of matrix metalloproteinases (MMPs).[6] According to both models, disruption of $\alpha_v\beta_6$-LAP binding would be expected to reduce TGF-β1 activity and thereby inhibit the tissue scarring associated with IPF.

Recently, a series of $\alpha_v\beta_6$ antagonists has been designed in our laboratories to mimic the RGD tripeptide motif found in LAP (Table 1).[7] Building on previous work, these peptidomimetics possess essential basic and acidic termini, linked by a spacer unit

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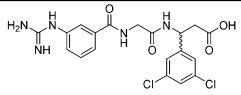
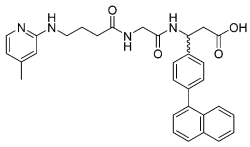
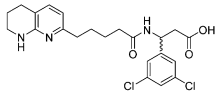
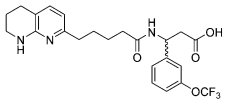
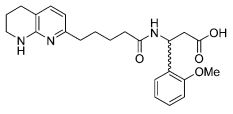
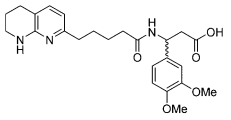
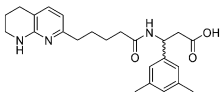
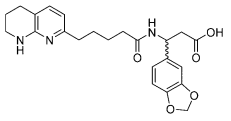
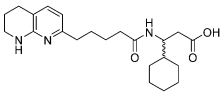
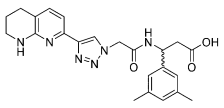
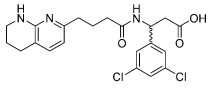
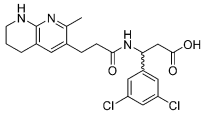
Table 1. Structures of the Integrin Antagonists Used in This Study Together with Their pIC_{50} and ED-Derived f_b Values

	Structure	pIC_{50}[a]	f_b (%)±SD[b]
1		7.2	38 ± 5
2		6.6	21 ± 1
3		6.6	27 ± 3
4		6.7	21 ± 4
5		<5.0	10 ± 4
6		6.5	13 ± 6
7		6.3	17 ± 6
8		6.6	15 ± 5
9		<5.0	3.8 ± 4
10		5.3	6.2 ± 5
11		<5.0	4.1 ± 5
12		<5.0	2.6 ± 5

[a]Data from cell adhesion assay; see ref 7. [b]Data from ED assay described in this paper. Mean f_b (%) from 4 repeats with standard deviation.

to simulate the Arg, Asp, and Gly amino acid residues, respectively.[8,9]

1 and **2** are established literature antagonists, **3**–**9** were previously described by us,[7] and **10**–**12** are reported here for the first time (see Supporting Information for synthesis and characterization). The ability of this series of compounds to inhibit $\alpha_v\beta_6$ activity was demonstrated by a cell adhesion assay.[7] Although able directly and reliably to detect inhibition of the $\alpha_v\beta_6$-LAP interaction, the assay requires access to, and maintenance of, a transfected human cell line overexpressing $\alpha_v\beta_6$. We therefore wished to construct a simple, rapid, and sensitive competitive assay to rank the order of $\alpha_v\beta_6$ target binding, and chose to examine the suitability of equilibrium dialysis (ED), with LC-MS detection, for this purpose.

ED employs a dialysis cartridge composed of two chambers separated by a semipermeable membrane (typically 10 kDa molecular weight cutoff). Equal volumes of analyte solution (dialysate) and protein solution are placed in each chamber (termed dialysate and assay chambers), and the system is allowed to reach equilibrium. Providing that the analyte passes freely through the membrane, and the protein does not, a measurable difference in analyte concentration between the two chambers may be attributed to binding of the analyte to the target protein. In cases where subsaturation concentrations of the analyte can be detected and quantified, it is possible to determine the dissociation constant (K_d) for the interaction by titration. ED has found widespread application in the quantification of drug–plasma protein binding[10−12] and drug–brain tissue interactions,[13] as part of ADME studies. The technique has also been used for examining the interactions of purified proteins, such as the binding of kringle 5 to glucose-regulated protein 78, which promotes tumor cell apoptosis.[14] Methods for analyte detection/quantification include radioactivity,[15] UV–visible spectroscopy,[16] and LC-MS.[17−19] Together with powerful analytical approaches, such as LC-MS, ED permits the study of competitive binding to plasma proteins using mixtures of ligands.[20] We wished to examine the feasibility of using ED with LC-MS detection to develop a competitive screening assay for $\alpha_v\beta_6$ antagonists.

As a preliminary experiment to establish the suitability of ED in the detection of $\alpha_v\beta_6$-antagonist binding, compound **1** (Table 1), a well characterized inhibitor of $\alpha_v\beta_6$,[9] was utilized. A buffered aqueous solution of **1** (1.06 μM, 25 μL) was dialyzed against either a solution of recombinant $\alpha_v\beta_6$ (0.27 μM, 25 μL) or an equal volume of buffer, as a control; see Supporting Information for experimental details. Quantitative LC-MS/MS analysis revealed that, in the control, **1** was equally distributed between the dialysate and assay chambers. In contrast, when $\alpha_v\beta_6$ was present in the assay chamber, a clear and measurable difference in the concentration of **1** was detected (see Figure 1). When expressed as the fraction of **1** bound to $\alpha_v\beta_6$ (f_b), a value of 20 ± 0.3% was obtained (control f_b = −0.5 ± 0.7%), which corresponded to 93 ± 3% saturation of the target integrin. Given that **1** is a nM inhibitor of $\alpha_v\beta_6$ (pIC_{50} = 7.2; cell adhesion assay),[7] this high degree of association was to be expected, and demonstrated that ED with LC-MS detection was capable of detecting the $\alpha_v\beta_6$-antagonist interaction.

Having established that ED was able to detect integrin–antagonist binding, we next sought to determine whether an ED-based assay could reveal binding preference in a competition between different antagonists. To that end, a solution containing a mixture of **1**, **2**, and **3** (0.53 μM each) was dialyzed against an equal volume of $\alpha_v\beta_6$ solution (0.53 μM). Compound **2** is an established integrin antagonist from Merck KGaA,[8] while **3** is a Nottingham-GSK compound.[7] The results, summarized in Table 1, showed a good correlation between the ED-determined f_b values and the pIC_{50} values of the three compounds. Inhibitors

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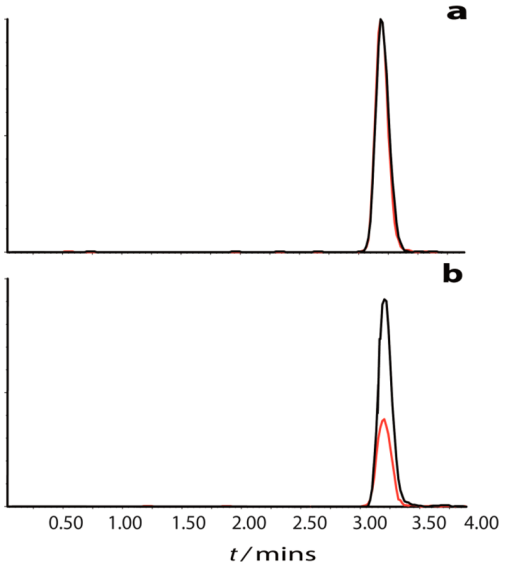

Figure 1. LC-MS/MS chromatograms for antagonist **1** detected in the dialysate (red) and assay chambers (black) of the ED cartridge in the absence (a) or presence (b) of $\alpha_v\beta_6$. Data are derived from the transition of $[M + H]^+$ m/z 452.3 → m/z 162.1, and they show a differential in the concentration of **1** between the two ED chambers in (b), which results from binding of **1** to $\alpha_v\beta_6$.

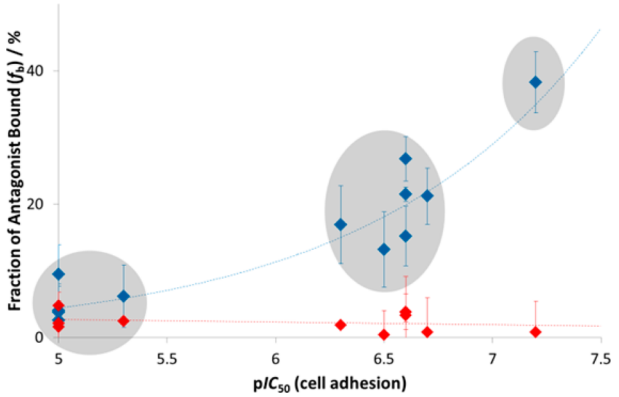

Figure 2. Plot of fraction of antagonist bound (f_b) in the competitive ED assay vs pIC_{50} for compounds **1**−**12** ED experiments conducted in the presence (blue) and absence (red) of $\alpha_v\beta_6$. pIC_{50} values were determined for individual compounds using the cell adhesion assay.

2 and **3**, which possessed identical pIC_{50} values of 6.6, also exhibited relatively similar f_b percentages. Compound **1**, with the highest pIC_{50}, gave the highest f_b. It was noted that **1** did not fully displace **2** and **3**, despite the equimolar ratio of each compound relative to $\alpha_v\beta_6$. Presumably this is due to the relatively narrow range of binding affinities exhibited by the three antagonists. It was envisaged that significantly weaker binders would show little or no bound fraction when in competition with these high affinity compounds.

Given that the competitive ED measurements described above showed promise in revealing relative $\alpha_v\beta_6$-antagonist binding properties, we constructed a more complex mixture of compounds (see Table 1), which exhibited a wider range of affinities. A solution of **1**−**12** (0.28 μM each, 25 μL) was dialyzed against an equal volume of $\alpha_v\beta_6$ (0.53 μM). After 24 h the distribution of each compound within the ED cartridge was determined by LC-MS (see Supporting Information Table S3 and Figure S6). A plot of f_b vs pIC_{50} is shown in Figure 2. These data revealed that the five compounds (**5**, **9**, **10**, **11**, and **12**) with pIC_{50} values < 5.5 possessed f_b values indistinguishable from the control and, thus, within experimental error, were unable to bind $\alpha_v\beta_6$ when in competition with higher affinity antagonists. Although not specifically added for this purpose, one or more of these compounds would make suitable controls for testing other potential antagonists, by acting as internal standards. The remaining seven compounds, which all had pIC_{50} values > 6.2, showed binding in the ED assay. Compound **1** was found to be the best $\alpha_v\beta_6$ binder, followed by a group of compounds with relatively similar affinities, which was comprised of **2**, **3**, **4**, **6**, **7**, and **8**.

Structure−activity relationships for compounds **3**−**9** are described elsewhere.[7] Triazole spacers between Arg and Asp mimetics having micromolar inhibitory activity for $\alpha_v\beta_3$ and $a_v\beta_5$ are precedented,[21] but introduction of a triazole spacer in this series as in **10** causes at least a 10-fold drop in activity against $\alpha_v\beta_6$ (cf. **7**). The approximate distance between the Arg and Asp mimetics is likely to be similar between **10** and **7**, so it may be the steric bulk or introduction of the nitrogen atoms that is detrimental. Chain shortened and/or reorientation of the naphthyridine ring (**11** and **12**) also lead to loss in activity, and this is likely due to the extended conformation of these compounds being too short to span the distance between the Arg and Asp binding sites in $\alpha_v\beta_6$.[7]

The results presented here demonstrate that ED is able to detect antagonist binding to $\alpha_v\beta_6$ integrin. A competitive assay with (at least) 12 components is able to reveal the relative binding affinities based on f_b values. A good correlation between the ED measurements and an established cell adhesion assay was obtained. The ED assay, although not directly detecting inhibition of the $\alpha_v\beta_6$-LAP complex, has the advantage of tolerating mixtures of ligands, and−potentially−small libraries of drug candidates. Moreover, $\alpha_v\beta_6$ is a commercially available protein, as are other integrins, and the ED assay does not require access to modified cell lines, which together make this approach for integrin antagonist discovery more widely available to those without the resources for cell-based methods. The major advantage of the competitive ED-LC-MS approach lies in its ability to rapidly identify the most potent binder from a mixture of potential antagonists, and we foresee that it is this property that will be exploited to access promising leads.

■ ASSOCIATED CONTENT

Supporting Information

Full experimental procedures, analytical conditions, and calibrations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

IPF, idiopathic pulmonary fibrosis; TGF-β1, transforming growth factor-β1; LAP, latency associated peptide; ED, equilibrium dialysis; LC-MS, liquid chromatography−mass spectrometry; MS/MS, tandem mass spectrometry; f_b, fraction bound

■ REFERENCES

(1) Scullion, J. E. Living with Idiopathic Pulmonary Fibrosis. *Chron. Respir. Disease* **2011**, *8*, 223−225.

(2) Fell, C.; Martinez, F. J.; Liu, L. X.; Murray, S.; Han, M. K.; Kazerooni, E. A.; Gross, B. H.; Myers, J.; Travis, W. D.; Colby, T. V.; Toews, G. B.; Flaherty, K. R. Clinical Predictors of a Diagnosis of Idiopathic Pulmonary Fibrosis. *Am. J. Respir. Crit. Care Med.* **2010**, *181*, 832−837.

(3) Raghu, G.; Collard, H. R.; Egan, J. J.; Martinez, F. J.; Behr, J.; Brown, K. K.; Colby, T. V.; Cordier, J.-F.; Flaherty, K. R.; Lasky, J. A.; et al. An Official ATS/ERS/JRS/ALAT Statement: Idiopathic Pulmonary Fibrosis: Evidence-based Guidelines for Diagnosis and Management. *Am. J. Respir. Crit. Care Med.* **2011**, *183*, 788−824.

(4) Raghu, G.; Weycker, D.; Edelsberg, J.; Bradford, W. Z.; Oster, G. Incidence and prevalence of idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* **2006**, *174*, 810−816.

(5) Ryan, R. M.; Mineo-Kuhn, M. M.; Kramer, C. M.; Finkelstein, J. N. Growth factors after neonatal type II alveolar epithelial cell proliferation. *Am. J. Physiol.* **1994**, *266*, 17−22.

(6) Tatler, A. L.; Jenkins, G. TGF-β activation and lung fibrosis. *Proc. Am. Thorac. Soc.* **2012**, *9*, 130−136.

(7) Adams, J.; Anderson, E. C.; Blackham, E. E.; Chiu, Y. W. R.; Clarke, T.; Eccles, N.; Gill, L. A.; Haye, J. J.; Haywood, H. T.; Hoenig, C. R. Structure Activity Relationships of α_v Integrin Antagonists for Pulmonary Fibrosis By Variation in Aryl Substituents. *ACS Med. Chem. Lett.* **2014**, *5*, 1207 DOI: 10.1021/ml5002079.

(8) Goodman, S. L.; Hölzemann, G.; Sulyok, G. A. G.; Kessler, H. Nanomolar Small Molecule Inhibitors for $\alpha_v\beta_6$, $\alpha_v\beta_5$, and $\alpha_v\beta_3$ Integrins. *J. Med. Chem.* **2002**, *45*, 1045−1051.

(9) Ruminski, P. G.; Lindmark, R. J.; Russell, M. A.; Clare, M.; Collins, P. W.; Rico, J. G.; Rogers, T. E.; Desai, B. N. WO9708145, Mar 6, 1997.

(10) McMenamy, R. H.; Oncley, J. L. The Specific Binding of L-Tryptophan to Serum Albumin. *J. Biol. Chem.* **1958**, *233*, 1436−1447.

(11) Sterling, K.; Rosen, P.; Tabachnick, M. Equilibrium Dialysis Studies of the Binding of Thyroxine by Human Serum Albumin. *J. Clin. Invest.* **1962**, *41*, 1021−1030.

(12) Waters, N. J.; Jones, R.; Williams, G.; Sohal, B. Validation of a rapid Equilibrium Dialysis Approach for the Measurement of Plasma Protein Binding. *J. Pharm. Sci.* **2008**, *97*, 4586−4595.

(13) Wan, H.; Rehngren, M.; Giordanetto, F.; Bergström, F.; Tunek, A. High-throughput Screening of Drug-Brain Tissue Binding and in Silico Prediction for Assessment of Central Nervous System Drug Delivery. *J. Med. Chem.* **2007**, *50*, 4606−4615.

(14) Davidson, D. J.; Haskell, C.; Majest, S.; Kherzai, A.; Egan, D. A.; Walter, K. A.; Schneider, A.; Gubbins, E. F.; Solomon, L.; Chen, Z.; Lesniewski, R.; Henkin, J. Kringle 5 of Human Plasmoinogen Induces Apoptosis of Endothilial and Tumor Cells through Surgace-Expressed Glucose-Regulated Protein 78. *Cancer Res.* **2005**, *65*, 4663−4672.

(15) Cressina, E.; Chen, L.; Abell, C.; Leeper, J. J.; Smith, A. G. Fragment Screening against the Thiamine Pyrophosphate Riboswitch *thiM*. *Chem. Sci.* **2011**, *2*, 157−165.

(16) Rabbani, A.; Abdosamadi, S.; Sari-Saraf, N. Affinity of anticancer drug, danomycin, to core histomes in solution: comparison of free and cross-linked proteins. *Acta Pharmacol. Sin.* **2007**, *28*, 731−737.

(17) van Liempd, S.; Morrison, D.; Sysmans, L.; Nelis, P. Development and Validation of a Higher-Throughput Equilibrium Dialysis Assay for Plasma Protein Binding. *J. Lab. Autom.* **2011**, *16*, 56−67.

(18) Kochansky, C. J.; McMasters, D. R.; Lu, P.; Koeplinger, K. A.; Kerr, H. H.; Shou, M.; Korzekwa, K. R. Impact of pH on Plasma Protein Binding in Equilibrium Dialysis. *Mol. Pharmaceutics* **2008**, *5*, 438−448.

(19) Zamek-Gliszczynski, M. J.; Ruterbories, K. J.; Ajamie, R. T.; Wickremsinhe, E. R.; Pothuri, L.; Rao, M. V. S.; Basavanakatti, V. N.; Pinjari, J.; Ramanathan, V. K.; Chaudhary, A. K. Validation of 96-well Equilibrium Dialysis with Non-radiolabeled Drug for Definitive Measurement of Protein Binding and Application to Clinical Development of Highly-Bound Drugs. *J. Pharm. Sci.* **2011**, *100*, 2498−2507.

(20) Wan, H.; Rehngren, M. High-throughput Screening of Protein binding by Equilibrium Dialysis combined with Liquid Chromatography and Mass Spectrometry. *J. Chromatogr., A* **2006**, *1102*, 125−134.

(21) Trabocchi, A.; Menchi, G.; Cini, N.; Bianchini, F.; Raspanti, S.; Bottoncetti, A.; Pupi, A.; Calorini, L.; Guarna, A. Click-Chemistry-Derived Triazole Ligands of Arginine-glycine-Aspartate (RGD) Integrins with a Broad Capacity to Inhibit Adhesion of Melanoma Cells and Both in Vitro and in Vivo Angiogenesis. *J. Med. Chem.* **2010**, *53*, 7119−7128.

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