Conformational mAb as a Tool for Integrin Ligand Discovery

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ABSTRACT

$\alpha_4\beta_1$ -Integrin (very late antigen-4 (VLA-4)) mediates cell adhesion to cell surface ligands (VCAM-1). Binding of VLA-4 to VCAM-1 initiates rolling and firm adhesion of leukocytes to vascular endothelium followed by the extravasation into the tissue. VLA-4-dependent adhesion plays a key role in controlling leukocyte adhesive events. Small molecules that bind to the integrin ligand-binding site and block its interaction with natural ligands represent promising candidates for treatment of several diseases. Following a flow cytometric screen for small molecule discovery, we took advantage of a conformationally sensitive anti- β_1 -integrin antibody (HUTS-21) and a small LDV-containing ligand (LDV-FITC) with known affinity to study binding affinities of several known and recently discovered integrin ligands. We found that binding of the LDV-containing small molecule induced exposure of HUTS-21 epitope and that the EC_{50} for antibody binding was equal to previously reported K_d for fluorescent LDV (LDV-FITC). Thus,

binding of HUTS-21 can be used to report ligand-binding site occupancy. We studied binding of two known integrin ligands (YLDV and TR14035), as well as of two novel compounds. EC_{50} values for HUTS-21 binding showed good correlation with K_d s determined in the competition assay with LDV-FITC for all ligands. A docking model suggests a common mode of binding for the small molecule VLA-4 ligands. This novel approach described here can be used to determine ligand-binding affinities for unlabeled integrin ligands, and can be adapted to a high-throughput screening format for identification of unknown integrin ligands.

INTRODUCTION

Integrins are cell surface receptors that mediate cell to cell, or cell to extracellular matrix adhesion. They play a major role in the regulation of immune cell recruitment to inflamed endothelia and sites of inflammation. Integrins participate in antigen-presenting cell-lymphocyte interactions, retention and mobilization of immature progenitors in the bone marrow, cancer cell trafficking, metastasis, and other events. They represent a target for several existing drugs for treatment of inflammatory diseases, antiangiogenic therapy, and antithrombotic therapy. Integrin ligands can also be used as imaging tools.^{1–4} Integrin-dependent adhesion avidity is regulated by a number of conformational changes of the protein. These can occur without a significant change in the expression levels of the molecules. Conformational changes include an increase in the affinity of the ligand-binding pocket, and others, that consists of the unbending (extension) of the integrin, along with hybrid domain swing, as

ABBREVIATIONS: BIO-1211, 4-((N'-2-methylphenyl)ureido)-phenylacetyl-L-leucyl-L-aspartyl-L-valyl-L-proline; HSA, human serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDV-containing small molecule, 4-((N'-2-methylphenyl)ureido)-phenylacetyl-L-leucyl-L-aspartyl-L-valyl-L-prolyl-L-alanyl-L-alanyl-L-lysine; LDV-FITC-containing small molecule, 4-((N'-2-methylphenyl)ureido)-phenylacetyl-L-leucyl-L-aspartyl-L-valyl-L-prolyl-L-alanyl-L-alanyl-L-lysine-FITC; mAb, monoclonal antibody; LIBS, ligand-induced binding sites; MCF, mean channel fluorescence (equivalent of mean fluorescence intensity); MCS, maximum common substructure; NIH MLSMR, NIH Molecular Libraries Small Molecule Repository; PDB, protein databank; VCAM-1, vascular cell adhesion molecule 1, CD106; VLA-4, very late antigen-4, CD49d/CD29, $\alpha_4\beta_1$ -integrin.

well as integrin “leg” separation.⁵ Recent data suggest that at least some of these conformations are regulated independently from the others.^{6,7} Conformational changes can be detected using conformationally sensitive antibodies, which bind to defined epitopes exposed only in certain molecular conformations. Some of these are known to be induced by the binding of the ligand (so-called ligand-induced binding sites (LIBS)).⁸ Several antibody epitopes have been mapped to the part of the very late antigen-4 (VLA-4) integrin surface between α - and β_1 -subunits, which is hidden in the resting, low affinity state because of the close subunit proximity, and exposed upon activation and/or ligand binding.^{9,10} The integrin conformation with exposed epitopes is attributed to the high-affinity activation state in one model of integrin activation and the ligand occupied conformation according to another.⁵ However, despite differing opinions about the role of epitope exposure, they represent a valuable tool for monitoring integrin conformations using a conventional flow cytometer.

Discovery of new small molecules that bind to the integrin ligand-binding site and block interaction with its natural ligand is part of the ongoing drug discovery process.^{2,11} The ability to detect specific binding of the ligand and determine its binding affinity is critical for these approaches. In this case a desirable assay would be if the binding of the unlabeled small molecule could be detected in a homogeneous assay. Here we describe a novel approach for the detection of the ligand-binding affinity based upon induction of ligand-induced epitopes. Using commercially available conformationally sensitive monoclonal antibodies (mAbs), we were able to confirm induction of ligand-induced epitopes as well as ligand-binding affinity for two previously described VLA-4 integrin ligands. EC_{50} values for the conformational mAb binding showed a good correlation with K_i s determined in the competitive binding assay with a well-characterized fluorescent ligand. We have also determined binding constants for two novel VLA-4 ligands, and verified them using a competitive binding assay. Ligands that induce activation epitopes may formally be referred to as agonists if they also induce intracellular signaling. This current approach can be extended to other integrins, and can be adapted for a high-throughput flow cytometry format.¹²

MATERIALS AND METHODS

Materials

The VLA-4-specific ligand^{13–15} 4-((*N'*-2-methylphenyl)ureido)-phenylacetyl-L-leucyl-L-aspartyl-L-valyl-L-prolyl-L-alanyl-L-alanyl-L-lysine (LDV) and its FITC-conjugated analog (LDV-FITC) were synthesized at Commonwealth Biotechnologies (Richmond, VA). Mouse anti-human CD29, HUTS-21(PE), isotype control (mouse IgG2a κ PE) clone G155-178 were purchased from BD Biosciences (San Jose, CA) and used according to manufacturer's instructions.

N-(2,6-Dichlorobenzoyl)-(L)-4-(2',6'-bis-methoxyphenyl)phenylalanine (TR14035) compound¹⁶ was synthesized by Dr. Wei Wang (Department of Chemistry, University of New Mexico). Two recently identified VLA-4 ligands 3-(adamantane-1-carboxylamino)-3-(4-ethoxyphenyl) propanoic acid and 3-(adamantane-1-carboxylamino)-3-(4-propoxyphenyl) propanoic acid (SID: 14732971, CID: 5197400; and SID: 14732972, CID: 4329131) were from NIH molecular libraries small molecule repository (NIH MLSMR) (<http://pubchem.ncbi.nlm.nih.gov/>) curated by BioFocus/DPI (South San Francisco, CA). All other reagents were from Sigma-Aldrich (St. Louis, MO). Stock peptide solutions were prepared in DMSO, at concentrations ~1,000-fold higher than the final concentration. Usually, 1 μ L of stock solution was added to 1 mL of cell suspension yielding a final DMSO concentration of 0.1%. Control samples were treated with equal amount of pure DMSO (vehicle).

Cells

The human histiocytic lymphoma cell line U937 was purchased from ATCC (Manassas, VA). Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ and 95% air in RPMI 1640 (supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μ g/mL streptomycin, 10 mM HEPES, pH 7.4, and 10% heat-inactivated fetal bovine serum). Cells were then harvested and resuspended in 1 mL of HEPES buffer (110 mM NaCl, 10 mM KCl, 10 mM glucose, 1 mM MgCl₂, 1.5 mM CaCl₂, and 30 mM HEPES, pH 7.4) containing 0.1% human serum albumin (HSA) and stored on ice. Cells were counted using the Coulter Multisizer/Z2 analyzer Beckman Coulter (Miami, FL). For experiments, cells were suspended in the same HEPES buffer at 10⁶ cells/mL and warmed to 37°C for 10 min prior to binding experiments (see below).

LDV-FITC Competitive Binding Assay

Cells in HEPES buffer containing 1 mM MgCl₂, 1.5 mM CaCl₂ were preincubated with different concentration of compounds, 1 μ M unlabeled LDV (control) or DMSO (vehicle), for 20–30 min at room temperature. Next, LDV-FITC was added to the cells/compound mix (10 nM final concentration), and cells were incubated for additional 30–40 min. FITC fluorescent (FL1 channel) was measured using BD FACScan flow cytometer collecting 5,000 events. The data were plotted as LDV-FITC-specific binding versus the concentration of competitor and the data were fitted to a one site competition equation. The equilibrium dissociation constant, K_d , was calculated using Cheng and Prusoff equation (K_d for LDV-FITC ~12 nM, labeled ligand concentration ~10 nM).

HUTS-21 Antibody Binding

U937 cells were suspended in the HEPES buffer (see earlier) 1 $\times$ 10⁶ cells/mL, 100 μ L aliquots (10⁵ cells) were incubated with

different concentrations of unlabeled compounds for 10 min. Next, 20 μ L of PE-labeled HUTS-21 antibodies was added and cells were incubated for additional 30–40 min at room temperature. Next, cells were washed with 1 mL of HEPES buffer, resuspended in 300–500 μ L of buffer, and analyzed by flow cytometry (FL2 channel, BD FACScan). The data were plotted as mean channel fluorescence versus the concentration of the compound. The data were fitted to a sigmoidal dose–response equation. To determine a level of nonspecific binding, cells were stained in parallel with the isotype control antibodies.

Statistical Analysis

Curve fits, statistics, and K_i calculations were performed using GraphPad Prism version 4.00 for Windows, GraphPad Software (San Diego, CA), www.graphpad.com. Each experiment was repeated at least two times. The experimental curves represent the mean of two or more independent runs. Standard error of the mean was calculated using GraphPad Prism.

Discovery of VLA-4 Ligands; The Flow Cytometric Screen for VLA-4 is Reported in PubChem

In brief, in 384-well plates prepared with Jurkat cells and delivered by HyperCyt platform to the flow cytometer, we could discriminate cell autofluorescence, the 2-fold increase of fluorescence of ligand bound to resting cells, and an additional 2-fold increase in ligand binding when the cells were treated with Mn^{2+} , which increased the affinity of VLA-4 as well as ligand binding. The Z' for the screen of ~25,000 compounds was ~0.7 (AIDs 528, 529, 576, 702, 703). While the screen was intended to identify allosteric ligands for VLA-4, small molecules that blocked LDV-FITC binding either in the presence or in the absence of Mn^{2+} were identified as competitive inhibitors at both 4°C and 37°C.

Post-screening analysis of the inhibitors detected in primary screening was performed by using MESA Analytics & Computing

clustering software package (Santa Fe, New Mexico). The MDL 320 fingerprint keys were used to represent the chemical compounds. Clustering at various similarity thresholds was done in order to select the most appropriate similarity threshold. The most potent inhibitor clusters were selected for follow-up. The most potent inhibitors (MLS000085920, MLS000044001, and MLS000085916, see Fig. 1) formed the basis of virtual screening of MLSMR library. The maximum common substructure (MCS) for the selected cluster was used as a query in virtual screening of the compound library. The most similar compounds were identified and the compounds were subsequently retested in dose response in the LDV-FITC dose–response assay described earlier. The compounds with the best activity are also included in Figure 1, MLS000521558, and MLS000521553.

Homology Modeling and Docking

To verify a common mode of binding to the VLA-4-binding site, we built a homology model for the headpiece of the VLA-4 integrin, which comprises the β -propeller from the α -subunit and the I-like domain from the β -subunit. The homology model was built using the SWISS-MODEL server,^{17–19} based on the X-ray structure of $\alpha V\beta 3$ -integrin complexed with a ligand containing the RGD motif (Protein Data Bank, <http://www.rcsb.org>, PDB access code: 1L5G). For sequence alignment, the T-coffee program^{20,21} was used and the model obtained was further refined manually to avoid deletions or insertions in the conserved regions. Finally, manganese ions (MIDAS, ADMIDAS, and LIMBS) have been added into the VLA-4-binding site with the same atomic coordinates as in the $\alpha V\beta 3$ structure. At the final step, the model has been minimized to reduce the steric clashes of the side chains without changing the backbone of the integrin.

Docking studies were carried out using FRED (OpenEye Scientific Software, Santa Fe, USA, FRED, <http://www.eyesopen.com/products/applications/fred.html>), which uses a precomputed

FIG. 1. Structure of molecules identified in screening and virtual screening. Maximum common substructure (MCS) is highlighted in bold.

database of conformations for a given ligand. Multi-conformer databases were generated using OMEGA (OpenEye Scientific Software, Santa Fe, USA, Omega, <http://www.eyesopen.com/products/applications/omega.html>) with default parameters that produced an average of 150 conformers per ligand. Docking simulations were performed with default parameters, and each ligand conformer was rigidly minimized based on shape and chemical complementarity to the protein-binding site. The grid-box was defined by increasing the size of RGD crystallized ligand by 5 Å on each side of the ligand. This procedure was found suitable to allow a number of compounds to fit into the binding site.

RESULTS

We have reported in PubChem a screen using a fluorescent ligand for VLA-4 in a homogeneous flow cytometry assay that identified novel inhibitors of VLA-4. Because of the relatively low signal background ratio of that assay (~2/1), we have explored alternatives for detecting and characterizing small molecule interactions. Ligand-induced binding sites (LIBS) are antibody epitopes that become exposed after a conformational change within an integrin molecule due to ligand binding. LIBS can be detected using conformationally sensitive mAbs that are commercially available for several integrins. We hypothesized that LIBS could quantitatively report the occupancy of the integrin ligand-

binding pocket by an unlabeled integrin ligand. To verify this idea, we have employed several small VLA-4 ligands with both previously reported and unknown binding affinities (Fig. 2).

An LDV small molecule (and its fluorescent analog, LDV-FITC (Fig. 2A)) is well-characterized VLA-4 ligand that has been used in several laboratories to detect VLA-4 affinity and conformational changes.^{6,13,22,23} Here, we used LDV-FITC as a labeled competitor to determine binding affinities (K_i) of other unlabeled ligands. The unlabeled LDV was used both as positive control for the induction of LIBS as well as a blocking compound to detect the level of non-specific binding in the competitive binding assay. The YLDV compound has an additional tyrosine residue between its N-terminal "cap" and LDV sequence (Fig. 2B). This modification decreased its binding affinity ~100-fold (Fig. 4A). TR14035 (Fig. 2C) is described in the literature as a potent ligand that blocks VLA-4 binding to vascular cell adhesion molecule 1 (VCAM-1).^{11,16,24} Compounds D and E (Fig. 2D and 2E) are two novel VLA-4 ligands discovered in a high-throughput screen at the University of New Mexico Center for Molecular Discovery (<http://pubchem.ncbi.nlm.nih.gov>, AID: 529, 702).

Binding of an Unlabeled LDV-Containing Small Molecule Induces Exposure of HUTS-21 Epitope With EC_{50} Identical to K_d for LDV-FITC Binding

The design of the LDV-containing small molecule (Fig. 2A) was based upon the published structure of BIO-1211 (Biogen) compound, which has been shown to induce LIBS.²⁵ The small changes in the structure of the molecule (two alanine and one lysine residues added in a region suggested by SAR to be outside of the binding pocket¹³) have not altered its ability to induce LIBS.

As shown in Figure 3A, the binding of conformationally sensitive mAbs was well behaved. Flow cytometric histograms were symmetrical and histogram peaks shifted to the right with increasing unlabeled ligand concentration. The signal to background ratio was about 10/1. Binding of isotype control mAbs was identical to the binding of HUTS-21 in the absence of the ligand (data not shown). The histogram showing binding of HUTS-21 at 0.1 nM LDV is at the same level as nonspecific mAb binding. Thus, in the absence of the ligand no HUTS-21 epitope exposure was observed. The concentration-dependent dose response for LIBS

FIG. 2. Structure of compounds used in the study. (A) 4-((N-2-methylphenyl)ureido)-phenylacetyl-L-leucyl-L-aspartyl-L-valyl-L-prolyl-L-alanyl-L-alanyl-L-lysine, LDV compound, based upon BIO-1211.^{13,25} (B) YLDV compound, additional L-tyrosine is shown in bold. (C) N-(2,6-dichlorobenzoyl)-(L)-4-(2',6'-bis-methoxyphenyl)phenylalanine, TR14035.¹⁶ (D) Recently identified VLA-4 ligand, 3-(adamantane-1-carboxylamino)-3-(4-ethoxyphenyl) propanoic acid, SID: 14732971, CID: 5197400; Compound D. (E) VLA-4 ligand, 3-(adamantane-1-carboxylamino)-3-(4-propoxyphenyl) propanoic acid, SID: 14732972, CID: 4329131, Compound E.

FIG. 3. Binding of HUTS-21 to U937 cells in the presence of different concentrations of LDV. **(A)** Cells were incubated with the indicated concentration of LDV in the presence of an excess of HUTS-21 mAbs, washed, and fluorescence was measured (5,000 events were collected). No specific binding of HUTS-21 was detected in the absence of LDV ligand (binding of isotype control Abs was identical to binding of HUTS-21 in presence of 0.1 nM LDV). Experiments were performed as described under Materials and Methods. **(B)** Mean fluorescence intensity plotted versus concentration of LDV (data replotted from **A**). The data were fitted using the sigmoidal dose-response equation using GraphPad Prism software. EC_{50} for the binding of HUTS-21 is essentially identical to previously reported K_d for LDV-FITC under these ionic conditions.¹³ Each point represents mean $\pm$ SEM of two independent determinations. One representative experiment out of three independent experiments is shown.

correlated well with ligand occupancy for LDV-FITC small molecule for both low- and high-affinity states of VLA-4 (Fig. 3B, for low affinity and²⁶ for high affinity). The EC_{50} for the induction of epitope exposure was identical to the previously published dissociation constant (K_d) for the fluorescent LDV analog (LDV-FITC).¹³ Thus, quantitatively the number of ligand occupied binding sites is reflected in the number of bound mAbs and LIBS sites. As the total concentration of VLA-4 receptors in solution was <0.1 nM, these experiments were performed under conditions at which no significant ligand depletion has been observed. Taken together, these data suggest that the LIBS dose response reflects the binding affinities for unlabeled integrin.

Two Known VLA-4 Ligands Compete With LDV-FITC for Binding to VLA-4 and Induce HUTS-21 Epitope Exposure Similar to an LDV-Containing Small Molecule

Next, we took advantage of LIBS detection to examine the binding of two previously characterized unlabeled VLA-4 ligands (Fig. 2B and 2C). Whereas YLDV is of low affinity, TR14035 (Tanabe, Fig. 2C)^{11,16} is reported to be a highly potent inhibitor of VLA-4 binding to VCAM-1.²⁴ Although this compound is reported as non-selective $\alpha_4\beta_1/\alpha_4\beta_7$ -ligand, since U937 cells do not express significant amounts of the β_7 -integrin subunit, the data presented here can be interpreted in terms of binding to VLA-4. To characterize the binding affinities of these compounds, we performed

competitive equilibrium binding experiments (Fig. 4A). Cells were incubated in the presence of 10 nM LDV-FITC with increasing concentrations of each compound. The resulting sigmoidal dose-response curves were fitted using the Cheng-Prusoff equation to determine K_i (which is analogous to a dissociation constant K_d) from the EC_{50} (Fig. 4A).

In parallel, HUTS-21-binding experiments were performed (Fig. 4B, analogous to Fig. 3B). A saturating amount of LDV (1 μ M LDV $\gg K_d$ (12 nM)) was used as a positive control. The resulting curves were fitted to a sigmoidal dose-response binding equation, in which the Hill slope was generally close to 1.0. EC_{50} s for the induction of HUTS-21 epitope exposure were similar to K_i values determined in a competition experiment (compare Fig. 4A and 4B). Introduction of the additional tyrosine into LDV sequence lowered the binding affinity of the compound by

about two orders of magnitude (compare EC_{50} for LDV and YLDV in Figs. 3 and 4).²⁵ Also, as shown for other VLA-4 ligands, the binding of TR14035 was strongly dependent upon the presence of divalent ions (Mn^{2+}) (data not shown), and its binding affinity was similar to the previously published IC_{50} for ligand binding.¹⁶ Thus, as for LDV, YLDV and TR14035 resulted in the LIBS exposure, and the occupancy of the ligand-binding pocket is reported by HUTS-21 antibody binding.

Two Novel Compounds Compete for LDV-FITC Binding and Induce HUTS-21 Epitope Exposure

Recently, through a screen based on LDV-FITC binding and virtual screening follow-up, we have identified a number of compounds that inhibit LDV-FITC binding (PubChem BioAssay AID# 529, 702). Two of the selected compounds (D and E, Fig. 2D and 2E) in the presence of 0.5 mM Mn^{2+} exhibited nanomolar affinity in an LDV-FITC competitive binding assay (EC_{50} = 90 nM leading to $K_i \sim 20$ nM) (AID #702 confirmatory, concentration-response relationship). To verify that these novel compounds involved the same mechanism of binding interaction, we evaluated the LIBS response in the absence of Mn^{2+} . The two VLA-4 ligands showed similar affinities in both the LDV-FITC competition and the HUTS-21-binding assays (Fig. 5). Once again, EC_{50} values for HUTS-21 binding correlated with K_i s determined in the competition assay with LDV-FITC.

FIG. 4. Competition between two known VLA-4 ligands and LDV-FITC ligand, and their effect upon HUTS-21 epitope exposure. **(A)** Competitive binding of LDV-FITC ligand to U937 cells in the presence of different concentrations of YLDV (—○—) or TR14035 (—●—). Experiments were performed as described under Materials and Methods. Data were normalized assuming that average fluorescence for nonspecific binding of 10 nM LDV-FITC (sample blocked with 1 μM unlabeled LDV) is equal to 0, and unblocked sample fluorescence (10 nM LDV-FITC with no competitor added) is equal to 1.0. The data were fitted using the one site competition equation using GraphPad Prism software. K_i values shown in the panel were calculated using the Cheng and Prusoff equation using K_d for LDV-FITC $\sim 12 \text{ nM}$, labeled ligand concentration (LDV-FITC) $\sim 10 \text{ nM}$. Each point represents mean $\pm$ SEM of two independent determinations. A representative experiment out of two independent experiments is shown. **(B)** Binding of HUTS-21 to cells in the presence of different concentrations of YLDV (—○—) or TR14035 (—●—). Cells were incubated with the indicated compound concentration in the presence of an excess of HUTS-21 mAbs, washed, and fluorescence was measured (experiment was performed as indicated in the legend for Fig. 3). Data were normalized with the relative fluorescence for nonspecific binding of HUTS-21 (isotype control, or sample without integrin ligand added) set to 0, and with the positive control sample fluorescence (1 μM unlabeled LDV) set to 1.0. The mean fluorescence intensity plotted versus concentration of compounds was fitted using the sigmoidal dose-response equation using GraphPad Prism software. EC_{50} s for binding are shown next to the curves. Each point represents mean $\pm$ SEM of two independent determinations. A representative experiment out of two independent experiments is shown.

Correlation Between EC_{50} for HUTS-21 Binding and K_i Determined in the Competition Assay

The data are summarized as a plot of EC_{50} s for HUTS-21 binding vs. K_i s determined in the competition assay with LDV-FITC (Fig. 6). A strong correlation between K_i and EC_{50} is observed for the five ligands studied. Thus, conformationally sensitive anti- β_1 -integrin mAbs can be used to determine binding affinities of unlabeled VLA-4 integrin ligands.

Docking Results

Docking experiments were carried out with the compounds D, E, and TR14035 as described in Materials and Methods to evaluate the modes of binding. The ligands share a similar trend in the docking model in that they interact with both integrin subunits, establishing hydrophobic interactions with aromatic residues from the β -propeller (α -subunit, Fig. 7, green surface) and hydrogen bonds with the I-like domain in the β -subunit (Fig. 7,

gray surface). Also, in all docking models, the ligands' carboxylic acid groups form coordinate covalent bonds to the manganese ion of the MIDAS center (Fig. 7, green sphere). In the TR14035-bound model, the 2,6-dimethoxybiphenylic group is oriented toward the α -subunit, being placed into a hydrophobic pocket defined by three aromatic residues: Phe214, Tyr187, and Trp188. The propoxy and ethoxy groups of the D and E compounds point to the same pocket, although in their case the hydrophobic interactions are not as strong as for TR14035 compound. This is the only type of interaction observed between the ligands and the α -subunit.

The fragment of the I-like domain embedded in the binding site contains mainly polar amino acids, thus a hydrogen bond network is observed in this area. The hydroxyl groups of the Ser152 and Ser154 side chains interact with carboxylic functions presented in all three ligands and form hydrogen bonds. An extra hydrogen bond is formed between the amidic moiety of compounds D and E and carbonyl group of Asn244 from the integrin backbone. In TR14035 model, the amidic group is in a good orientation toward Asn244 but is located too far away

from it for the interaction to be possible. The adamantyl substituents from the compounds D and E and the 2,6-dichlorobenzene ring from TR14035 are situated in a hydrophobic pocket close to the residues Tyr153 and Cys207. Another common interaction between VLA-4 and ligands is the coordination by carboxylic group of manganese ion of the MIDAS center. This coordination together with the hydrogen bonds helps to stabilize the ligand in the binding site.

DISCUSSION

Integrins are a family of extracellular adhesion receptors that represent one of the modern therapeutic targets for multiple human diseases. In particular, α_4 -integrins expressed on a variety of white blood cells are implicated in the pathogenesis of asthma, rheumatoid arthritis, inflammatory bowel diseases, and others.^{3,11} VLA-4/VCAM-1 interaction plays a role in the homing, retention, and mobilization of hematopoietic progenitors and stem cells in bone marrow.^{27,28} Vascular integrins play a role in tumor

FIG. 5. Competition between novel VLA-4 ligands and LDV-FITC ligand, and their effect upon HUTS-21 epitope exposure. The experimental setup is analogous to the experiment shown in Figure 3. **(A)** Competitive binding of LDV-FITC ligand to U937 cells in the presence of different concentrations of Compound D (—○—) or Compound E (—●—) (Fig. 2D and 2E). **(B)** Binding of HUTS-21 to cells in the presence of different concentrations of Compound D (—○—) or Compound E (—●—) (Fig. 2D and 2E). Each point represents mean $\pm$ SEM of two independent determinations. A representative experiment out of two independent experiments is shown.

angiogenesis.¹ Therefore, at least a dozen pharmaceutical companies are actively pursuing the development of α_4 -integrin ligands that block VCAM-1 binding but do not induce cell activation.¹¹

Previously, it has been reported that several different integrin ligands can “activate” integrin molecules. This activation is often described as “outside-in” signaling associated with integrin cross-linking after multivalent ligand binding. In a series

FIG. 6. EC_{50} for HUTS-21 binding (determined in the HUTS-21-binding experiments as shown in Fig. 4B and Fig. 5B) plotted versus K_i (determined in the competition assay with LDV-FITC as shown in Fig. 4A and Fig. 5A) for the five compounds studied. Letters next to the experimental points indicate compounds as shown in Figure 2. Each point represents mean $\pm$ SEM of two independent experiments, performed in duplicate. The data were fitted using the linear regression equation and a coefficient of determination (R^2) was calculated using GraphPad Prism software.

of recent studies, binding of monovalent LDV-containing ligands did not lead to time-dependent affinity changes, even at saturating ligand concentrations. In contrast, cellular activation through G-protein-coupled and several other receptors led to integrin affinity up-regulation.^{6,13,29} However, the exposure of HUTS-21 mAb epitope, used in the current study, was dependent on ligand binding rather than cell activation. HUTS-21 binding can be detected in the presence of low ligand concentration for the high-affinity state of the ligand-binding pocket (activated integrin), or it required a higher ligand concentration for the low-affinity state (ie, the resting or inactive integrin conformation). “Inside-out” activation through G-protein-coupled receptors had no direct effect upon HUTS epitope expo-

sure. Thus, ligand binding by itself may mechanically induce a series of conformational changes, since ligand-induced binding of HUTS-21 can be observed on ice.²⁶ Thus, the exposure of the HUTS epitope appears to reflect a ligand-induced binding site (LIBS).

Determination of Ligand-Binding Affinity for the Unlabeled Ligand

Because the binding of our ligands to VLA-4 induces a conformational change, which can be detected using integrin-specific antibodies, integrins represent a unique system where fractional occupancy of the ligand-binding pocket can be assessed using conformationally sensitive mAbs. Moreover, this can be done using an unlabeled ligand, and thus, compounds can be tested over a wide range of concentrations. The other advantage is that because of antibody specificity the same compound can be used to study its binding to different integrins (as multiple integrins are reported to have shared ligands.³⁰). Thus, this technology is ideal for use in a screen for novel compounds that have a quality that is unique for integrin ligands, namely to cause ligand-induced conformational change. However, compounds that induce the conformational change may be considered agonists that have the potential to activate integrin signaling, and could exhibit less desirable side effects.

An alternative approach is a competition assay in which the unknown compound is competing against labeled ligand (fluorescent for the case of flow cytometry). In this case the major problem is that the affinity of the unlabeled compound in the primary screen

FIG. 7. Docking of compounds TR14035, D, and E to a homology model for the headpiece of the VLA-4 integrin in the VLA-4-binding site. The β -propeller of the α_4 -subunit is shown as the green surface, the I-like domain in the β_1 -subunit is shown as the gray surface. Mn^{2+} ions are shown in CPK representation (ADMIDAS and LIMBS are colored in purple, MIDAS is colored in green).

is much lower than for the labeled ligand. Thus, a very high concentration of competitor is necessary. The other issue is ligand-binding specificity. After the compound is identified, testing for binding specificity is required. This is not an issue in the case of conformationally sensitive mAb-based assay, which has a very high degree of binding specificity. Thus, the technology presented allows the detection of ligand binding in a simple and efficient way. In our hands, the sensitivity of mAb detection with phycoerythrin is nearly an order of magnitude better than direct detection of ligand binding with fluorescein. The dynamic range of the response spans ligands with affinity varying over all least three orders of magnitude.

Furthermore, the mAb method is uniquely compatible with discrimination of ligands that induce the LIBS, as well as the detection of ligands that compete and do not induce LIBS. As of yet, we have not identified ligands that meet this criteria. The ability to detect ligand occupancy in a homogeneous assay without having to develop labeled ligand would facilitate primary and secondary screens for these novel ligands.

The results from these LIBS mAb-binding studies provide further evidence that these VLA-4 ligands bind to the integrin's ligand-binding pocket, thus directly competing with LDV-FITC rather than binding to an allosteric site. The docking studies suggest similar binding modes for the small molecules, although the increased affinity of TR14035 may result from its ability to interact with both integrin subunits.

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AUTHOR DISCLOSURE STATEMENT

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REFERENCES

1. Alghisi GC, Ruegg C: Vascular integrins in tumor angiogenesis: mediators and therapeutic targets. *Endothelium* 2006;13:113–135.
2. Tucker GC: Integrins: molecular targets in cancer therapy. *Curr Oncol Rep* 2006;8:96–103.
3. Vanderslice P, Woodside DG: Integrin antagonists as therapeutics for inflammatory diseases. *Expert Opin Investig Drugs* 2006;15:1235–1255.
4. Woodside DG, Vanderslice P: Cell adhesion antagonists: therapeutic potential in asthma and chronic obstructive pulmonary disease. *BioDrugs* 2008;22:85–100.
5. Arnaout MA, Goodman SL, Xiong JP: Structure and mechanics of integrin-based cell adhesion. *Curr Opin Cell Biol* 2007;19:495–507.
6. Chigaev A, Waller A, Zwartz GJ, Buranda T, Sklar LA: Regulation of cell adhesion by affinity and conformational unbending of alpha4beta1 integrin. *J Immunol* 2007;178:6828–6839.
7. Larson RS, Davis T, Bologa C, Semenuk G, Vijayan S, Li Y, et al.: Dissociation of I domain and global conformational changes in LFA-1: refinement of small molecule-I domain structure-activity relationships. *Biochemistry* 2005;44: 4322–4331.
8. Frelinger AL, III, Cohen I, Plow EF, Smith MA, Roberts J, Lam SC, et al.: Selective inhibition of integrin function by antibodies specific for ligand-occupied receptor conformers. *J Biol Chem* 1990;265:6346–6352.
9. Mould AP, Barton SJ, Askari JA, McEwan PA, Buckley PA, Craig SE, et al.: Conformational changes in the integrin beta A domain provide a mechanism for signal transduction via hybrid domain movement. *J Biol Chem* 2003;278:17028–17035.
10. Mould AP, Travis MA, Barton SJ, Hamilton JA, Askari JA, Craig SE, et al.: Evidence that monoclonal antibodies directed against the integrin beta subunit plexin/semaphorin/integrin domain stimulate function by inducing receptor extension. *J Biol Chem* 2005;280:4238–4246.
11. Jackson DY: Alpha 4 integrin antagonists. *Curr Pharm Des* 2002;8:1229–1253.
12. Sklar LA, Carter MB, Edwards BS: Flow cytometry for drug discovery, receptor pharmacology and high-throughput screening. *Curr Opin Pharmacol* 2007;7:527–534.
13. Chigaev A, Blenc AM, Braaten JV, Kumaraswamy N, Kepley CL, Andrews RP, et al.: Real time analysis of the affinity regulation of alpha 4-integrin. The physiologically activated receptor is intermediate in affinity between resting and Mn(2+) or antibody activation. *J Biol Chem* 2001;276:48670–48678.
14. Chigaev A, Zwartz G, Graves SW, Dwyer DC, Tsuji H, Foutz TD, Edwards BS, et al.: Alpha4beta1 integrin affinity changes govern cell adhesion. *J Biol Chem* 2003;278:38174–38182.
15. Chigaev A, Buranda T, Dwyer DC, Prossnitz ER, Sklar LA: FRET detection of cellular alpha4-integrin conformational activation. *Biophys J* 2003;85:3951–3962.

16. Egger LA, Kidambi U, Cao J, Van Riper G, McCauley E, Mumford RA, *et al.*: Alpha(4) beta(7)/alpha(4)beta(1) dual integrin antagonists block alpha(4)beta(7)-dependent adhesion under shear flow. *J Pharmacol Exp Ther* 2002;302:153–162.
17. Arnold K, Bordoli L, Kopp J, Schwede T: The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* 2006;22:195–201.
18. Guex N, Peitsch MC: SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* 1997;18:2714–2723.
19. Schwede T, Kopp J, Guex N, Peitsch MC: SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Res* 2003;31:3381–3385.
20. Notredame C, Higgins DG, Heringa J: T-Coffee: a novel method for fast and accurate multiple sequence alignment. *J Mol Biol* 2000;302:205–217.
21. Poirot O, O'Toole E, Notredame C: Tcoffee@igs: A web server for computing, evaluating and combining multiple sequence alignments. *Nucleic Acids Res* 2003;31:3503–3506.
22. Chigaev A, Waller A, Amit O, Sklar LA: GalphaS-coupled receptor signaling actively down-regulates alpha4beta1-integrin affinity: a possible mechanism for cell de-adhesion. *BMC Immunol* 2008;9:26.
23. Hyduk SJ, Chan JR, Duffy ST, Chen M, Peterson MD, Waddell TK, *et al.*: Phospholipase C, calcium, and calmodulin are critical for alpha4beta1 integrin affinity up-regulation and monocyte arrest triggered by chemoattractants. *Blood* 2007;109:176–184.
24. Gong Y, Barbay JK, Dyatkin AB, Miskowski TA, Kimball ES, Prouty SM, *et al.*: Synthesis and biological evaluation of novel pyridazinone-based alpha4 integrin receptor antagonists. *J Med Chem* 2006;49:3402–3411.
25. Lin K, Ateeq HS, Hsiung SH, Chong LT, Zimmerman CN, Castro A, *et al.*: Selective, tight-binding inhibitors of integrin alpha4beta1 that inhibit allergic airway responses. *J Med Chem* 1999;42:920–934.
26. Chigaev A, Waller A, Amit O, Halip L, Bologa CG, Sklar LA: Realtime analysis of conformation-sensitive antibody binding provides new insights into integrin conformational regulation. *J Biol Chem* 2009;284:14337–14346.
27. Lapidot T, Petit I: Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. *Exp Hematol* 2002;30:973–981.
28. Lapidot T, Dar A, Kollet O: How do stem cells find their way home?. *Blood* 2005;106:1901–1910.
29. Hernandez-Hansen V, Smith AJ, Surviladze Z, Chigaev A, Mazel T, Kalesnikoff J, Lowell CA, *et al.*: Dysregulated FcepsilonRI signaling and altered Fyn and SHIP activities in Lyn-deficient mast cells. *J Immunol* 2004;173:100–112.
30. Humphries JD, Byron A, Humphries MJ: Integrin ligands at a glance. *J Cell Sci* 2006;119:3901–3903.

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