Small Molecule Agonist of Very Late Antigen-4 (VLA-4) Integrin Induces Progenitor Cell Adhesion

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Background: Activation of integrins may improve cell retention rates in stem cell transplantation.

Results: The first small molecule agonist of integrin α4β1 is generated and enhances cell adhesion mechanisms in vitro.

Conclusion: The agonist binds at the subunit interface, inducing ligand binding with consequent displacement of compound.

Significance: The agonist may improve progenitor cell retention as an adjunct to cell-based therapy.

Activation of the integrin family of cell adhesion receptors on progenitor cells may be a viable approach to enhance the effects of stem cell-based therapies by improving cell retention and engraftment. Here, we describe the synthesis and characterization of the first small molecule agonist identified for the integrin α4β1 (also known as very late antigen-4 or VLA-4). The agonist, THI0019, was generated via two structural modifications to a previously identified α4β1 antagonist. THI0019 greatly enhanced the adhesion of cultured cell lines and primary progenitor cells to α4β1 ligands VCAM-1 and CS1 under both static and flow conditions. Furthermore, THI0019 facilitated the rolling and spreading of cells on VCAM-1 and the migration of cells toward SDF-1α. Molecular modeling predicted that the compound binds at the α/β subunit interface overlapping the ligand-binding site thus indicating that the compound must be displaced upon ligand binding. In support of this model, an analog of THI0019 modified to contain a photoreactive group was used to demonstrate that when cross-linked to the integrin, the compound behaves as an antagonist instead of an agonist. In addition, THI0019 showed cross-reactivity with the related integrin α4β7 as well as α5β1 and αLβ2. When cross-linked to αLβ2, the photoreactive analog of THI0019 remained an agonist, consistent with it binding at the α/β subunit interface and not at the ligand-binding site in the inserted (“I”) domain of the αL subunit. Co-administering progenitor cells with a compound such as THI0019 may provide a mechanism for enhancing stem cell therapy.

Clinical trials have shown that stem/progenitor cell therapy for cardiovascular indications is well tolerated and may improve heart function (1, 2). However, low rates of cell retention and engraftment after cell delivery may be problematic in achieving maximal benefits from the therapy. Studies have shown that fewer than 10% of cells are retained in the heart within hours after intracoronary administration of bone marrow progenitor cells in patients who have had a myocardial infarction (3). Despite low retention rates, recent studies in animal models of myocardial infarction (4) and in patients with dilated cardiomyopathy (5) have indicated that the early retention of transplanted stem cells directly correlates to improved functional outcomes. Thus, finding a means to increase cell retention would lead to more robust cell therapy (6).

One approach to increase cell retention has been to target the integrin family of adhesion receptors expressed on the surface of stem and progenitor cells (6). Integrins are a family of 24 distinct heterodimeric cell surface receptors composed of α and a β subunit (7). Integrins mediate the adhesion of cells to extracellular matrix proteins or to ligands expressed on the surface of neighboring cells. They have been validated as drug targets in humans in that inhibition of integrin function has led to the development of therapeutics for cardiovascular and autoimmune indications (8, 9). Half of the α subunits contain an extra “inserted” or “I” domain, which is responsible for ligand binding. In those integrins that lack an I domain, the binding site includes regions of both the α and β subunits (10). Integrin activity depends on the coordination of divalent cations at the metal ion-dependent adhesion site (MIDAS). A primary determinant of ligand binding is the coordination of the cation at the MIDAS site by an acidic residue supplied by the ligand itself (11). Thus, most peptide or small molecule competitive antagonists of integrin function contain an acidic group that interacts at the MIDAS site (10). Integrin activity also depends on conformation; integrins exist in an equilibrium between different conformational states that dictate their relative affinity for ligand (12).

The abbreviations used are: MIDAS, metal ion-dependent adhesion site; VLA-4, very late antigen-4; EPC, endothelial progenitor cell; HPC, hematopoietic progenitor cell; VCAM-1, vascular cell adhesion molecule-1; CS1, connecting segment-1; LIBS, ligand-induced binding site; SDF-1, stromal cell-derived factor-1; MAdCAM-1, mucosal addressin cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1.

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* This work was supported, in whole or in part, by National Institutes of Health Grant 2 T32-CA-09598-21 (to W. S. B.). P. V., R. J. B., and C. W. G. are named on a patent application that was submitted by the Texas Heart Institute.

† This article contains supplemental Movies 1–4.
The up-regulation of integrin activity or expression on the surface of progenitor cells has been used to increase the retention of progenitor cells both in vitro and in disease models in vivo. These approaches have targeted integrins either directly by using activating antibodies (13) or indirectly by genetically manipulating or preconditioning cells with recombinant proteins (14–16). Targeting key integrins directly with a small molecule agonist may be an effective means to enhance cell retention in stem cell therapy. An attractive target for such an approach is the integrin α4β1, which is expressed on the surface of several progenitor cell types, including endothelial progenitor cells (EPCs) and hematopoietic progenitor cells (HPCs) (17, 18). α4β1 has been shown to be critical for progenitor cell homing to sites of ischemia, as well as for cell fusion, in animal models of stem cell therapy (17, 19). The predominant ligands for α4β1 are vascular cell adhesion molecule-1 (VCAM-1) and the alternatively spliced connecting segment-1 (CS1) sequence of fibronectin (7). Importantly, expression of both VCAM-1 and fibronectin is up-regulated after tissue injury, including after a myocardial infarction (20, 21).

One way in which integrins are activated physiologically is through outside-in signaling via ligand binding; this was initially demonstrated when small RGD peptides that bind αIIbβ3 were found to be partial agonists, as well as competitive antagonists, of cell adhesion (22). More recently, a small molecule antagonist of αLβ2 was found to be a partial agonist of cell adhesion under low affinity conditions (23). These findings led us to hypothesize that we can convert a known integrin antagonist to a full agonist. In this study, we describe the generation and characterization of the first known small molecule agonist of α4β1, an integrin that lacks an I domain. This small molecule agonist promotes α4β1-mediated cell adhesion, rolling, spreading, and migration. As such, it has significant effects on stem/progenitor cell adhesion under both static and flow conditions and therefore may prove to be a useful adjunct to stem cell therapy.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cell Lines**—The synthesis of THI0019, THI0003 (also known as TBC3567), TBC3486, and THI0455 has been previously described (24–26). For all assays described, THI0019 was dissolved in DMSO to make a 1 mM stock solution, and dilutions were made in assay buffer or media to yield the desired final working concentrations in 1% DMSO (vehicle). Synthesis of BIO5192 and its methyl ester has been previously described (27). Human collagen types II and IV, serum fibronectin, and vitronectin were purchased from Sigma. Human VCAM-1, MadCAM-1 Fc chimera, ICAM-1, and SDF-1 were purchased from R&D Systems (Minneapolis, MN). The CS1-BSA conjugate has previously been described (28) and was synthesized at New England Peptide (Gardner, MA). Antibodies were purchased from the following: ABD Serotec (Raleigh, NC) (HP2/1 (anti-α4) and 38 (anti-αL)); R&D Systems (Minneapolis, MN) (P5D2 (anti-β1) and BBIG-11 (anti-ICAM-1)); Invitrogen (SAM-1 (anti-α5), anti-MadCAM-1, and donkey anti-goat-647); Pharmingen (FIB27 ((anti-B7), HUTS-21 (anti-β1), WM59 (anti-CD31), 581 (anti-CD34), 89106 (anti-VEGFR2), and 9EG7 (anti-β1)); BD Biosciences (L25 (anti-α4)); Millipore (Temecula, CA) (B44 (anti-β1)); and Santa Cruz Biotechnology (Santa Cruz, CA) (WW-9 (anti-Flk-1), C-20 (goat polyclonal anti-α4)). The anti-VCAM-1 mAb P3C4 and anti-β1 mAb TS2/16 were purified from hybridomas purchased from the Developmental Studies Hybridoma Bank at University of Iowa and American Type Culture Collection, respectively. mAb 33B6 and the HPB-ALL T cell line were gifts from B. McIntyre (M. D. Anderson Cancer Center, Houston, TX). The cell lines Jurkat, K562, human umbilical vein endothelial cells, M2-10B4, HSB, and TF-1 were obtained from American Type Culture Collection (Manassas, VA) and were maintained in recommended culture media. The mutant Jurkat cell line not expressing α4 integrin (Jurkat (α4−)) (29) was a gift from Dr. David Rose, University of California at San Diego, La Jolla, CA.

**Generation of EPCs**—Human EPCs were generated from peripheral blood essentially as described previously (13). Buffy coats from human donors were obtained from Gulf Coast Regional Blood Center, Houston, TX, and were separated over a Ficoll gradient to isolate the mononuclear cell layer. Cells were plated in tissue culture flasks coated with human fibronectin (Sigma) in EC basal medium-2 (Clonetics, San Diego) supplemented with EGM-2 SingleQuots. After 4 days, nonadherent cells were removed by washing with phosphate-buffered saline. Fresh medium was added, and adherent cells were expanded in culture for 5 weeks. Endothelial progenitor cells were characterized by flow cytometry for surface expression of CD34, CD133, Flk-1 (KDR), CD31, and the α4 subunit of VLA-4. For confocal microscopy, cells were seeded onto a collagen-coated coverslip for 3 h at 37 °C, 5% CO2 and were incubated with 2 μg/ml Alexa Fluor 488-conjugated acetylated LDL (Invitrogen) in complete medium for 1 h at 37 °C. Cells were fixed in 2% paraformaldehyde and were stained with 4 μg/ml lectin from *Ulex europaeus*-Atto 594 conjugate (Sigma) for 1 h at room temperature. Images were captured on a Leica TCS SPE confocal fluorescent microscope (Mannheim, Germany) by using Leica Application Suite Advanced Fluorescence software version 2.5.2-6939.

**Static Cell Adhesion Assays**—Ligands (CS1-BSA, VCAM-1, MadCAM-1, fibronectin, ICAM-1, vitronectin, and collagen I and IV) in 50 μl of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl (TBS) were added to wells of a 96-well plate and allowed to coat overnight at 4 °C. To maximize the window to evaluate agonist activity, a suboptimal coating concentration of ligand was used. This ligand concentration corresponded approximately to that which would yield ≤5% adhesion as determined by dose-response curves of ligand binding to the appropriate cell type. For assays in which both antagonism and agonism effects were to be measured (e.g. Fig. 10, D, E, G, and H), the concentration of ligand corresponded to that which would yield roughly 50% adhesion. Exact ligand concentrations were as follows: Fig. 1, B, 0.03 μg/ml CS1-BSA, and C, 0.3 μg/ml CS1-BSA; Fig. 2B, 0.6 μg/ml CS1-BSA; Fig. 3, A, 0.2 μg/ml CS1-BSA, C, 0.5 μg/ml VCAM-1, and E, 0.1 μg/ml VCAM-1; Fig. 6, A and B, 1 μg/ml VCAM-1; Fig. 9, A, 1 μg/ml MadCAM-1, C, 1 μg/ml fibronectin, and D, 5 μg/ml ICAM-1; and Fig. 10, C, 0.5 μg/ml VCAM-1, D and E, 3 μg/ml VCAM-1, F, 5 μg/ml ICAM-1, and G and H, 15 μg/ml ICAM-1. All assays were performed as described pre-
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Briefly, 2 × 10^6 cells were labeled for 30 min with calcein-AM (Molecular Probes), washed, resuspended in binding buffer, and added to ligand-coated plates (2 × 10^2 cells/well) that had been blocked with 2% BSA. After a 30-min incubation at 37 °C, the plates were washed three times with binding buffer; the adherent cells were lysed, and fluorescence was measured on a Tecan Safire² plate reader. Because of the high background adhesion of TF-1 cells, assays with this cell line were performed at room temperature. Standard curves were run for each assay to convert fluorescence units to cell number. For each assay, the cells expressed the appropriate integrin receptor either endogenously (Jurkat/α4β1, Jurkat/α2β1, EPC/α4β1, TF-1/α4β1, K562/α5β1, K562/α1β1, human umbilical vein endothelial cells/αvβ3, Jurkat (α4−)/αLβ2, and HSB/αLβ2) or in recombinant form (K562/α4β1, K562/α4β7, and K562/α1β1). Generation of the recombinant K562 cell lines has been described previously (31). The binding buffer was TBS with 1 mM MgCl₂ and 1 mM CaCl₂ for low affinity α4β1 assays or TBS with 1 mM MnCl₂ for high affinity α4β1 assays. For cells in which the α4β1 integrin was empirically determined to be in a very low affinity state (K562/α4β1 and EPCs), TBS with 1 mM MnCl₂ was used as the buffer. Cross-screening assays for α4β7/MAdCAM-1, α5β1/fibronectin, αvβ3/vitronectin, and α1β1/collagen IV were performed in TBS with 1 mM MnCl₂. Assays for αLβ2/ICAM-1 were conducted in TBS with 2 mM MgCl₂ and 5 mM EGTA. Assays for α2β1/collagen I were performed in TBS with 1 mM MgCl₂.

**FACS Analysis**—Jurkat, Jurkat (α4−), K562, K562(α4β1), or TF-1 cells (5 × 10⁶) were suspended in 100 μl of buffer (Tyrode’s containing 1 mg/ml glucose, 1 mM MgCl₂, and 1 mM CaCl₂). Primary mAb (10 μg/ml) was added, and cells were incubated for 1 h on ice. For LIBS experiments, vehicle or compounds were added at the same time as primary mAb. Cells were washed and resuspended in 50 μl of buffer containing FITC-conjugated GAM secondary antibody and were then incubated on ice for 30 min. After another three washes with buffer, cells were resuspended in 500 μl of buffer and were analyzed on a Beckman Coulter Epics XL-MCL.

**Parallel Plate Flow Detachment Assays**—Detachment assays were performed as described previously (30). Recombinant human VCAM-1 (10 μg/ml) (Fig. 3, G and H) or 5 μg/ml (Fig. 5E) in 0.1 mM NaHCO₃ (pH 9.5)) was immobilized overnight at 4 °C onto 24 × 50-mm slides cut from 15 × 100-mm polystyrene Petri dishes. The slides were washed with PBS, blocked with 2% (w/v) BSA for 2 h at room temperature, and assembled into a parallel plate flow chamber. For detachment assays, vehicle, 10 μM THI0019, 10 μg/ml mAb TS2/16, or combinations of each were mixed with Jurkat cells in low affinity running buffer, and then 2.0 × 10⁶ cells were injected into the flow chamber and allowed to settle on the slides for 10 min. An increasing linear gradient of shear flow was pulled over the adherent cells for 300 s with the use of a computer-controlled syring pump (Harvard Apparatus). Shear stress calculations were determined every 50 s. The shear stress in dynes/cm² is defined as (6 μL)/(wh²), where μ is the viscosity of the medium (0.007); Q is the flow rate in cm³/s; w is the width of the chamber (0.3175 cm), and h is the height of the chamber (0.01524 cm). The number of cells attached was recorded by digital microscopy (VI-470 charge-coupled device video camera; Optronics Engineering) at ×20 on an inverted Nikon DIAPHOT-TMD microscope every 50 s and was plotted against time.

**Cell Rolling Assays**—Stromal cells (M2-10B4) were seeded on 24 × 50-mm slides cut from 125-ml tissue culture flasks, cultured overnight under standard tissue culture conditions, and assembled to a parallel plate flow chamber. TF1 cells (2.0 × 10⁶) were mixed with vehicle or 10 μM THI0019 in running buffer and then injected into the parallel plate flow chamber system. A constant shear flow of 0.5 dynes/cm² was applied to the system for 300 s, and the TF1 cells rolling across the stromal cell monolayer were recorded by digital microscopy. The digital recordings were then analyzed by using the Imaris Bitplane software (version 7.6.1) to determine the velocity of individual cells moving across the monolayer. The viewing area is 500 μm, and only cells that traveled at least 400 μm were included. The velocity of a cell is defined as the distance traveled divided by the time to travel that distance.

**Cell Spreading Assays**—VCAM-1 (50 μl of 6 μg/ml) was coated in TBS overnight at 4 °C onto high binding 96-well plates (Costar). Plates were blocked with 2% BSA for 1 h at room temperature and were washed with complete media (RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin). HPB-ALL cells (10⁶ cells in complete media) were added to wells and incubated for indicated time points at 37 °C. Images of cells were captured at ×10 magnification on an Olympus IX71 inverted microscope (Olympus America, Inc., Center Valley, PA) equipped with an AHUO39020 CCD camera. For each treatment group, images from three separate wells were quantitated (four fields counted per well) in a blinded fashion, and the data were presented as the percentage of cells spreading. For confocal images, HPB-ALL cells were incubated on glass slides coated with VCAM-1 for 10 min. Cells were stained for actin in red (phalloidin) and α4 integrin in green (goat polyclonal anti-α4 C-20 followed by donkey anti goat-647). Nuclear staining is shown in blue (Hoechst 33342).

**Migration Assays**—Migration assays were performed in 3-μm pore size Transwells (24 wells, Costar, Cambridge, MA). The upper chambers were pre-coated with 3 μg/ml fibronectin or 10 μg/ml VCAM-1 in 50 μl of TBS overnight at 4 °C and were then blocked with 2% BSA for 1 h at room temperature. After washing with migration medium (RPMI 1640 medium supplemented with 1% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin), the upper chambers were loaded with Jurkat cells (2 × 10⁵ cells) in 160 μl of migration medium. The lower chambers contained 600 μl of migration medium supplemented with 10 μg/ml SDF-1α to induce chemotaxis. Jurkat cells were mixed with vehicle (1% DMSO) or THI0019 at the indicated concentrations immediately prior to being added to the upper chamber. After a 4-h incubation at 37 °C, 5% CO₂, the upper chambers were removed, and cells in the lower chamber were collected and counted on a hemocytometer. Results are expressed as the total number of cells migrated.

**In Silico Docking of THI0019 in α4β7 Crystal Structure**—Modeling Suite 2012 (Schrodinger LLC, New York) was used on a 16-core 2.4 GHz AMD Operon system to visualize THI0019 binding to an integrin model. A Glide (version 5.8) docking
model was generated starting with a crystal structure of α4β7. The structure 3v4v was downloaded from the Protein Data Bank (32) and read into a Maestro (version 9.3). All chains except A (α4) and B (β7) were deleted. A basic protein preparation was performed using program defaults, with the addition of filling in missing side chains and deleting water molecules beyond 5 Å from heteroatoms. Missing atoms were identified in Ser-559 in chain A and Cys-455 in chain B. The complete preparation of the protein portion of the model involved the following: 1) basic protein preparation; 2) assignment of heteroatom states; 3) H-bond assignment, including PROPKA; 4) deletion of waters with less than three H-bonds to non-waters; 5) Impref “H-only” minimization; and 6) Impref “Minimize All” to root mean square deviation 0.5. For THI0019, a Lig PREP (version 2.6) calculation was performed on the structure (imported as a SDF file) by using default criteria and Epik (version 2.3) ionization. A Glide Grid was generated using default settings based on the crystal structure ligand. In addition, a constraint was specified for metal ion. A virtual screening workflow was submitted for the crystal structure ligand and then for THI0019. The virtual screening workflow involved docking with Glide XP mode, starting with three extra conformations and rescoring with the Prime MMGBSA Gbind.

Cross-linking Experiments—Cells and buffers were selected to maximize the interaction of compounds with integrin. K562 (α4β1) cells or compounds in 150 μl of TBS (pH 7.4), 1 mM MnCl2 were placed in wells of a 48-well plate coated with 0.5% polyHEMA. Alternatively, cells were premixed with vehicle or 10 μM compound before being added to wells. Plates were placed in a SpectroLinker XL-1000 (Spectronics Corp., Westbury, NY) 4.5 cm from the UV source and exposed to UV radiation at 312 nm for 3 min. Cells, compounds, or mixtures were removed from the plates and transferred to 96-well plates (5 × 10^4 cells/well) for the static adhesion assay. We conducted the αβ2/ICAM-1 cross-linking studies in an identical manner, except that we used HSB cells (2 × 10^5 cells/well) in TBS (pH 7.4), 2 mM MgCl2, and 5 mM EGTA.

Statistical Analysis—An unpaired Student’s t test was used to determine statistical significance between treatment groups. Differences were considered significant at p < 0.05.

RESULTS
Integrin Antagonist Is Converted to an Agonist—TBC3486 is a potent (IC50 = 9 nM), selective antagonist of the integrin α4β1 (31) and was used as a template for the design of an α4β1 agonist (Fig. 1A). Using cell-based adhesion assays, we measured the extent of binding of the human T cell line Jurkat to wells coated with the CS1 sequence of fibronectin conjugated to BSA. We screened compounds at concentrations from 0.1 to 30 μM, a range in which TBC3486 completely abrogated cell adhesion under both high and low affinity conditions (Fig. 1, B and C). Subsequent conversion of the carboxylic acid group of THI0003 to a methyl ester resulted in the formation of a compound (THI0019) that showed agonist activity under both high and low affinity conditions (Fig. 1, B and C). Thus, we chose the compound THI0019 for further evaluation. It is important to note that modification of the key carboxylic acid residue to a methyl ester does not convert all classes of α4β1 antagonists to agonists.
agonists. For example, applying this same modification to the antagonist BIO5192 ($K_D = 10 \text{ pM}$) (33) resulted in the formation of a compound that retained its ability to inhibit $\alpha_4\beta_1$-mediated adhesion, albeit to a weaker extent than that of the parent compound (Fig. 2).

We performed subsequent adhesion assays by using suboptimal ligand concentrations to maximize the window for observing increases in cell binding. In cell adhesion assays in which Jurkat cells bound to CS1-conjugated BSA, THI0019 showed a dose-dependent enhancement in cell binding with an effective concentration giving half-maximal binding (EC$_{50}$) of 1.7 $\mu$M (Fig. 3A). In this assay format, the compound increased the number of cells bound to CS1 by 20–30-fold. However, THI0019 did not induce cell adhesion to a CS1-BSA conjugate in which the CS1 sequence had been modified by changing the key aspartic acid residue (LDV) to alanine (LAV). Furthermore, neutralizing antibodies to the $\alpha_4$ or $\beta_1$ integrin subunit blocked the adhesion-inducing effects of THI0019, whereas antibodies to the $\alpha_5$, $\alpha_L$, $\beta_7$, or $\beta_2$ integrin subunits had no effect (Fig. 3B). These findings indicated that the binding of the cells to CS1-BSA was integrin-dependent and that THI0019 did not induce adhesion to other regions of the CS1 sequence or to the BSA conjugate. When VCAM-1 was used as the ligand (Fig. 3C), THI0019 induced an even more pronounced 100-fold increase in Jurkat cell binding, with an EC$_{50}$ of 1.2 $\mu$M. However, this effect of THI0019 was not observed when using a mutant Jurkat cell line that does not express $\alpha_4$ integrins (Fig. 3C) or by pretreating Jurkat cells with antibodies to VCAM-1 or the $\alpha_4$ or $\beta_1$ integrin subunits (Fig. 3D).

To determine whether THI0019 had a similar effect on cells that are candidates for regenerative medicine, we isolated human EPCs from peripheral blood and characterized them by confocal microscopy and flow cytometry. The cells showed two hallmark characteristics of human EPCs as follows: acetylated low density lipoprotein uptake and $U. europaeus$ lectin binding (data not shown). Furthermore, the cells showed a heterogeneous expression of the progenitor cell marker CD34 and were positive for the endothelial markers CD31 and VEGFR2, and they had little or no expression of CD133, suggesting a late EPC phenotype (data not shown). Importantly, the cells were positive for $\alpha_4$ integrin. Similar to our findings with Jurkat cells, THI0019 induced a dose-dependent increase in EPC binding to VCAM-1 (EC$_{50}$ of 3.7 $\mu$M), increasing the number of cells bound by 10–30-fold above baseline (Fig. 3E). This effect was blocked by antibodies to the $\alpha_4$ or $\beta_1$ integrin subunits (Fig. 3F).

Cells Treated with THI0019 Are Resistant to Shear Stress—To determine whether THI0019 affects cells under conditions of shear stress, we evaluated Jurkat cells or EPCs in parallel plate flow chambers. Cells were mixed with either vehicle or 10 $\mu$M THI0019 and infused onto slides coated with VCAM-1. After allowing the cells to settle for 10 min, we determined the rate of cell detachment as the flow rate increased. Under these conditions, the calculated shear stress ranged from 0 to 45 dynes/cm$^2$. Jurkat cells treated with vehicle gradually detached with $\sim 25\%$ remaining at 15 dynes/cm$^2$, a rate approximating arterial shear stress (Fig. 3G). In contrast, Jurkat cells treated with THI0019 detached at a much slower rate, with 63% of cells still attached at 15 dynes/cm$^2$. The results were even more dramatic with EPCs (Fig. 3H). Vehicle-treated EPCs rapidly detached from the VCAM surface, most likely because of the low basal activity of the $\alpha_4\beta_1$ integrin expressed on EPCs compared with Jurkat cells. In contrast, THI0019-treated EPCs detached at a very slow rate, and most cells remained attached even under the highest level of shear stress tested.

THI0019 Does Not Induce Ligand-induced Binding Site Epitopes—Because of the short duration of the adhesion assays, we believe the enhanced cell adhesion was probably not due to an increase in cell surface expression of the integrin. To confirm this, we used flow cytometry to show that treatment of Jurkat cells with THI0019 for 30 min had no effect on the binding of the monoclonal antibodies (mAbs) HP2/1 and 33B6, which recognize the $\alpha_4$ and $\beta_1$ integrin subunits, respectively (Fig. 4A). Similar results were obtained after cells were incubated for 4 h with THI0019 before processing (data not shown).

To determine whether THI0019 alters integrin conformation, we used flow cytometry to examine the effect of the compound on the binding of a series of mAbs that recognize LIBS epitopes. Binding of ligand induces a conformational rearrangement in the $\beta_1$ subunit that results in the exposure of these epitopes and increased antibody binding. In a similar fashion, the antagonist TBC3486, a ligand mimic, has been shown to also increase binding of LIBS antibodies (31). Flow cytometry experiments (Fig. 4B) demonstrated that although TBC3486 induced the binding of the LIBS mAbs B44, HUTS-
21, and 9EG7, the agonist compound THI0019 did not increase or decrease the exposure of these epitopes relative to the vehicle control.

**THI0019 Is Synergistic with Mn<sup>2+</sup>/H11001 and Activating mAb TS2/16**—Mn<sup>2+</sup>/H11001 cation and the mAb TS2/16 both activate integrins experimentally but by different mechanisms. For integrins such as α<sub>4</sub>β<sub>1</sub>, Mn<sup>2+</sup> binds to the cation-binding sites in the β subunit and up-regulates integrin affinity (34). The epitope for activating TS2/16 is located in the β<sub>1</sub> subunit, and upon binding, TS2/16 induces a high affinity conformation of β<sub>1</sub> integrins (35). Because THI0019 did not induce major conformational changes in the β subunit, as shown by the LIBS analysis, we evaluated the effect of the compound when combined with these agents. For these experiments, we used a human K562 cell line engineered to express recombinant α<sub>4</sub> because the resulting α<sub>4</sub>β<sub>1</sub> integrin was empirically determined to be in a very low affinity state. Fig. 5, A and B, shows the dose-response curves of these cells adhering to the CS1 ligand. Cell binding was clearly enhanced in the presence of Mn<sup>2+</sup>/H11001 or TS2/16. If suboptimal concentrations of CS1 were used, either of these agents or THI0019 had little or no effect on cell adhesion (Fig. 5, C and D). However, when the cells were treated with a combination of THI0019 and Mn<sup>2+</sup>/H11001 or TS2/16, we observed a synergistic effect on cell adhesion. No increase in adhesion was seen when Mn<sup>2+</sup>/H11001 or TS2/16 was used in combination with vehicle or when BSA was used as substrate. These findings indicate that the mechanism of THI0019 agonism is different from that of Mn<sup>2+</sup>/H11001 or TS2/16.

The synergistic effect we observed under static conditions translated to increased adhesion under flow conditions. In parallel plate flow experiments using Jurkat cells, the detachment curves for cells treated with TS2/16 or THI0019 alone were shifted far to the right relative to those for vehicle-treated cells (Fig. 5E and supplemental Movies S1–S3). When THI0019 and

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**FIGURE 3.** THI0019 enhances binding of Jurkat and EPCs under both static and flow conditions. A, C, and E, dose-response curves showing the effects of THI0019 on binding of Jurkat cells to CS1-BSA containing either the wild-type LDV or a mutated LAV binding sequence (A). Jurkat cells or a mutant Jurkat line that does not express α<sub>4</sub> integrin (α<sub>4</sub><sup>-</sup>) to VCAM-1 (C), and EPCs to VCAM-1 (E). Results are expressed as the number of cells attached ± S.D. from triplicate wells. B, D, and F, specificity was determined by preincubating the cells with buffer (none) or antibodies (10 μg/ml) to integrin subunits or isotype control antibodies. *, p < 0.05, versus respective Ig controls. Cell detachment assays under conditions of flow were performed with Jurkat (G) or EPCs (H) and VCAM-1. Results are expressed as the mean percentage of cells attached ± S.D. from triplicate runs. *, p < 0.05, versus vehicle-treated cells.
TS2/16 were combined, few cells detached, even under very high shear stress (supplemental Movie S4).

**THI0019 Facilitates Cell Rolling, Spreading, and Migration**—
In static adhesion assays, the binding of the hematopoietic progenitor cell line TF-1 to VCAM-1 was greatly enhanced by THI0019 in an α4β1-dependent manner (Fig. 6, A and B). To further delineate the components of cell trafficking affected by the agonist, we performed rolling assays using these cells in parallel plate flow chambers. TF-1 cells were infused across the surface of M2-10B4 stromal cells expressing VCAM-1, and a constant shear flow of 0.5 dynes/cm² was applied. We analyzed video images to identify rolling cells (total of 200–600 events), and we determined the relative velocity of the rolling cells on the basis of the number of frames required for a cell to travel a specified distance. Of the rolling cells, a significantly higher percentage of THI0019-treated cells traveled at slower rates than vehicle-treated cells (Fig. 6C). This translated to a 27% decrease in the average velocity of the rolling cell population as a whole (Fig. 6D). As a decrease in velocity typically precedes cell arrest and firm attachment, these data indicate that the
agonist is facilitating early events in the cell extravasation process, including rolling.

To determine whether THI0019 promotes cell spreading, we incubated HPB-ALL cells with vehicle or THI0019 in 96-well plates coated with BSA or VCAM-1. HPB-ALL cells were used as α4β1-dependent spreading is readily detected in this cell type (36). Images were captured and quantitated at 30, 90, and 240 min (Fig. 7A). The cells did not spread on BSA but readily spread on VCAM-1. A significantly higher percentage of THI0019-treated cells spread at 30 and 90 min than did vehicle-treated cells, and the spreading was more robust (Fig. 7B). At 240 min, the percentage of cells that spread on VCAM-1 was similar between the two groups. In a qualitative assessment of cell spreading, HPB-ALL cells were incubated on glass slides coated with VCAM-1 and evaluated by confocal microscopy. Although overall spreading was accelerated in this format relative to the 96-well plate regardless of treatment, THI0019 promoted substantially more cell spreading than did vehicle treatment (Fig. 7C). Vehicle-treated cells were generally more rounded, whereas THI0019-treated cells were more elaborately spread with α4 integrin localized in some cases to the filopodia.

We next examined whether the THI0019-induced enhancement in cell adhesion and spreading promoted or adversely affected cell migration. Stromal cell-derived factor −1 (SDF-1) is a primary chemokine involved in progenitor cell trafficking, both in the bone marrow and in homing to sites of ischemia and inflammation (21, 37). Jurkat cells were treated with vehicle or THI0019 and placed in the upper compartment of a Transwell chamber in which the membranes were coated with either fibronectin or VCAM-1; SDF-1 was placed in the bottom chamber. The results of the assay were similar for both ligands (Fig. 8). SDF-1 induced migration of cells above background levels. When cells were treated with THI0019, the number of migrated cells increased significantly above that observed with SDF-1 alone. As typically seen in these assays, the positive effect on cell migration resulted in a bell-shaped dose-response curve. The optimal migration was reached at 0.1 μM THI0019. The extent of migration seen at the highest dose of THI0019 tested (10 μM) was not significantly different from that seen with SDF-1 alone.

**THI0019 Cross-reacts with Other Integrins**—THI0019 was derived from the antagonist TBC3486, which is highly selective for α4β1 (31). Because the agonist was active in the micromolar range, cross-reactivity with other integrins was possible. THI0019 had negligible effects on cell binding mediated by αvβ3, α1β1, and α2β1 (data not shown); however, it enhanced the binding of cells expressing the related integrin α4β7 to mucosal addressin cell adhesion molecule (MAdCAM)-1, with an EC_{50} similar to that previously measured for the binding of α4β1 to VCAM-1 (Fig. 9A). This effect was blocked by antibodies to α4, β7, and MAdCAM-1 but not β1 (Fig. 9B). Surprisingly, THI0019 also showed agonist activity in assays measuring α5β1- and αLβ2-mediated adhesion to fibronectin and intercellular adhesion molecule (ICAM)-1, respectively (Fig. 9, C and D). The latter was particularly unexpected because αL contains an I domain that is the primary determinant of integrin binding. Neutralizing antibodies against α4 did not inhibit these effects (Fig. 9, E and F). Using flow cytometry, we ensured that the cell lines used expressed little or no α4 integrin (data
not shown) so that any effects of possible cross-talk between α4 integrins and α5β1 or α1β2 would be minimized.

**THI0019 Overlaps with the Ligand-binding Site of α4 Integrins**—To further elucidate the mechanism by which THI0019 acts as an agonist of integrin activity, we docked the compound into the recently published x-ray crystal structure of α4β7 (38) because no crystal structure forms of α4β1 have been determined. The α4β7 integrin shares the same α subunit as α4β1, and, as described above, the agonist THI0019 enhanced the binding of α4β7-expressing cells to the ligand MAdCAM-1. THI0019 is a structural analog of the ligand mimetic TBC3486 and, not surprisingly, docked favorably into the ligand-binding site (ΔGbind score of −75.9) (Fig. 10A). The model predicts that THI0019 bridges both the α and β subunits, which is consistent with previous x-ray structures of integrins co-crystallized with small molecules (10, 38). Unlike most antagonists, however, there was no interaction with the MIDAS site in the β subunit, which is consistent with the LIBS antibody results. The model does predict hydrogen bonding between the amide NH group of residue Ser-238 of the β subunit with the carbamate carbonyl of the compound. In addition, the amide carbonyl and NH groups of Asn-235 are predicted to form hydrogen bonds with one of the urea NH groups and the ester carbonyl of the compound, respectively. There was clearly interaction between one of the thiophene rings and the α subunit, including π-π stacking interactions with residues Tyr-187 and Phe-214. Site-directed mutagenesis has shown that Tyr-187 is critical for α4β1 binding to VCAM-1 and CS1 (39) and for α4β7 binding to MAdCAM-1 (40). In addition, the analogous residue in the α5 subunit, Phe-187, is required for optimal binding of fibronectin to α5β1 (39). This may explain in part how THI0019 can interact with all three of these integrins. However, this binding mode results in the paradox that the binding site for the small molecule agonist overlaps with that of the ligand, at least for integrins that do not contain an I domain. As such, the compound must be displaced from the binding pocket upon ligand bind-

**FIGURE 7. THI0019 facilitates cell spreading on VCAM-1.** A, HPB-ALL cells were incubated with vehicle or THI0019 in wells coated with BSA or VCAM-1 for 30, 90, or 240 min. At each time point, images were captured, and representative images from each time point are shown. B, images were quantified, and the results are expressed as the mean percentage of cells that spread ± S.D. from triplicate wells (only the 240-min time point is shown for BSA control). *, p < 0.05, versus vehicle-treated cells. C, confocal images of vehicle-treated or THI0019-treated HPB-ALL cells incubated on glass slides coated with VCAM-1 for 10 min. Cells are stained for actin (red) and α4 integrin (green). Nuclear staining is shown in blue.
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FIGURE 8. THI0019 enhances SDF-1-mediated cell migration. Results of SDF-1-induced migration of Jurkat cells in Transwell chambers coated with fibronectin (FN) or VCAM-1. Cells were treated with THI0019 or vehicle (−) as indicated. After 4 h, the number of cells in the lower chamber were counted. Results are expressed as the total number of cells migrated ± S.D., *p < 0.05. versus SDF-1 alone.

One of the major hurdles to achieving maximal benefits from stem cell therapy is the low retention of administered cells at the target site. The integrin α4β1 plays a key role in the homing of progenitor cells to sites of injury and mediates cell adhesion to expressed ligands (17, 18). In this study, we describe the development of the first small molecule agonist of α4β1 or any integrin lacking an I domain. We generated this agonist, THI0019, by making two structural modifications to a known α4β1 antagonist. THI0019 enhanced rolling, spreading, adhesion, and migration of cells in an α4β1-dependent fashion. Although these studies were performed in vitro, our finding that THI0019 significantly increased the adhesion of EPCs and HPCs under both static and flow conditions suggests that this compound may potentially improve cell therapy by helping to home and retain cells at the site of injury.

There are similarities and key differences between THI0019 and previously described integrin agonists. Studies have shown that an RGD peptide can trigger activation of integrin αIβ3, which allows it to bind fibrinogen after washout of the peptide (22). Binding of the peptide induces a conformational change in the integrin that promotes binding to fibrinogen, possibly by facilitating recognition of synergy sequences present in the ligand. The RGD peptide also functions to inhibit integrin function, yet the partial agonist and antagonist effects are due to occupation of the same binding site (22). We speculate that similar events are applicable to THI0019, except that it is a full agonist. The small molecule agonist binds with relatively low affinity and induces a subtle conformational switch that facilitates ligand binding with consequent displacement of the compound. Such a conformation may promote recognition of accessory binding sites known to be present in VCAM-1 and fibronectin (42, 43).

Agonists have also been described for the β2 family of integrins (23, 44, 45), which contain I domains. A partial agonist of αLβ2 integrin has been identified that is an allosteric antagonist when screened in the presence of Mn2+ but functions as an agonist in the presence of Mg2+ and Ca2+ (23). The authors speculate that both activities are mediated by binding to the same pocket and that the interaction of the compound with the I domain is altered depending on whether Ca2+ or Mn2+ is bound. In the presence of Ca2+, the compound can bind to the I domain in a manner that induces an active conformation. By inducing sustained adhesion to substrate, the partial agonist effectively inhibited leukocyte migration by preventing detachment of the trailing edge of cells (23). In addition, two research groups have used high throughput screening to identify agonists of αMβ2 (44, 46). These compounds share common structural features and are thought to stabilize the high affinity conformation of the αMβ2 integrin (44, 45). Moreover, these compounds have been proposed for use as anti-inflammatory agents.
agents because they inhibit leukocyte migration by not allowing detachment of cells once bound firmly to their counter-receptors (44, 45). Proof-of-concept studies have demonstrated the efficacy of these compounds in animal models of peritonitis, nephritis, and coronary angioplasty (44, 45). In contrast, THI0019 promotes rather than inhibits cell migration. Therefore, THI0019 would be used clinically in a different manner. We believe it could be used as an adjunct to cell-based therapy; premixing cells with THI0019 immediately before injection may enhance the homing and engraftment of delivered cells, and potential mechanistic side effects would be minimized because the total dose injected would be low, thus limiting the circulating levels of free compound.

THI0019 is synergistic with two other known integrin activators, Mn²⁺ and mAb TS2/16, both of which interact with the β1 subunit. Thus, the agonist functions through a mechanism distinct from that of either Mn²⁺ or TS2/16. In addition, THI0019 does not induce epitopes recognized by three different LIBS mAbs, all of which bind the β subunit. This finding indicates that the compound does not engage the MIDAS site, which is consistent with the lack of a carboxylic acid group on THI0019 and THI0019’s inability to induce a global conformational change in the β subunit. Unlike other known integrin activators, THI0019 may induce subtle conformational changes, and the primary effects may be mediated through the β1 subunit. Previous molecular docking studies of its parent antagonist molecule, TBC3486, into a model of the β1 subunit indicate that the carboxylic acid group and surrounding motifs bind to the β1 subunit, leaving the bis(arylmethyl)aminocarbonyl group available to interact with the α subunit (47). Activity data from multiple analogs indicate that the bis(arylmethyl)aminocarbonyl motif of THI0019 is much more sensitive to

**FIGURE 9.** THI0019 enhances α4β7, α5β1, and αLβ2-mediated cell adhesion. A, C, and D, dose-response curves of THI0019-treated cells showing the binding of K562 (α4β7) cells to MAdCAM-1 (A), K562 cells to fibronectin (C), and mutant Jurkat (α4–) cells to ICAM-1 (D). Results are expressed as the number of cells attached ± S.D. from triplicate wells. B, E, and F, specificity was determined by preincubating the cells with buffer (none) or antibodies (10 μg/ml) to integrin subunits or isotype controls. *, p < 0.05, versus respective Ig controls.
structural modifications than is the rest of the molecule. Docking of the compound into the recently published crystal structure of THI0019 predicts that although portions of THI0019 interact with the β subunit through hydrogen bonding, one of the key binding determinants is through π-π stacking of the thiophene rings with Tyr-187 and Phe-214 in the α4 subunit. These two residues have recently been shown to interact with a class of small molecule antagonists of α4β7 that were co-crystallized with the integrin (38). A precedent for the importance of the interaction with the α subunit was recently shown in studies of RUC-1, a small molecule antagonist of integrin αIIbβ3 that has a unique mode of binding (48). X-ray crystal structure data indicate that RUC-1 binds exclusively to the α subunit with only minor bridging to the β subunit through water molecules. In contrast to antagonists that are ligand mimetics that bind and induce a high affinity conformation, RUC-1 appears to bind and maintain the integrin in a closed low affinity conformation. Similar to THI0019, RUC-1 does not induce LIBS epitopes in the β subunit and has activity in the low micromolar range.

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probably because it does not engage in a high affinity interaction with the MIDAS site of the integrin (48). Unlike THI0019, however, RUC-1 is an antagonist of integrin function and does not induce ligand binding. Further studies, including co-crystallization of the integrin with the compound, will be required to delineate the details of the interaction between the agonist THI0019 and the integrin.

The mode of binding predicted by our docking experiments indicates that THI0019 binds at a site that overlaps the ligand binding pocket. Thus, we hypothesized that the compound would have to be displaced from this site upon ligand binding. To test this prediction, we synthesized a structural analog of THI0019 in which one of the thiophene rings was replaced with a photoreactive phenyl azide. If the sites overlapped, cross-linking of the compound to the integrin would inhibit rather than enhance cell adhesion. Mixing the cells with the analog compound followed by UV treatment converted the compound from an agonist to an antagonist, thus supporting our model. Ironically, this suggests that if a compound binds to the integrin with too high affinity, thereby preventing its displacement by ligand, it would not be able to function as an agonist. This idea is supported by the fact that additional analoging did not result in a compound that was significantly more potent than THI0019. This may also be the reason why the weak antagonist THI0003 (the transition compound between TBC3486 and THI0019) was a suitable intermediate to generate an agonist. In contrast, neither TBC3486 nor BIO5192, which contains a diphenyl urea motif that confines potent binding to α4β1 (49), could be converted directly to a full agonist by simply esterifying the carboxylic acid group.

THI0019 shows agonist activity against integrin α4β1 and α4β7 with an EC50 value in the 1–2 μM range. At these concentrations, it is not uncommon to detect cross-reactivity with other integrins (50, 51). In contrast to the potent parent compound, TBC3486, which was highly selective for α4β1, THI0019 also regulates adhesion mediated by α5β1 and αLβ2. Small cyclic RGD peptides have been identified previously that inhibit both α4β1 and α5β1 with IC50 values in the low micromolar range (50). Furthermore, regions of the β-propeller domain of the α5 subunit contain extensive homology to the α4 subunit, including the region surrounding residue Tyr-187. In fact, mutations to the analogous residue in α5 significantly reduce adhesion of α5β1 to fibronectin (39). Assuming a similar binding mode, any ligand (or ligand mimetic)-induced activation of the integrin by the agonist may also apply to α5β1.

The activity toward αLβ2 was somewhat surprising because this integrin contains an I domain in the α subunit. This is not without precedent because small molecule integrin antagonists have been identified that inhibit both α4β1 and αLβ2 at micromolar concentrations (51). If THI0019 occupies a site in αLβ2 that is analogous to that of α4β1, it would not be predicted to overlap with the ICAM-1-binding site, which is located in the I domain. Rather, THI0019 may bind to the β-propeller domain of the αL subunit and influence the orientation and therefore the activity of the I domain. The results of the cross-linking studies support this hypothesis. When THI0455 was cross-linked to α4β1, it competed with VCAM-1 for the ligand-binding site, behaving as an antagonist. In the case of αLβ2, however, THI0455 remained an agonist even after cross-linking, as predicted if binding occurs at the αβ interface and not within the ligand-binding site of the I domain. Previously, an epitope mapping study of antibodies that interact with the I domain of αLβ2 showed that antibodies that were antagonists of the integrin bound in proximity to the ICAM-1 contact site, whereas an antibody that had agonist activity bound distal to the ligand-binding site, closer to where the I domain contacts the β-propeller domain (52). These cross-target activities may enhance the ability of THI0019 to promote the retention of stem/progenitor cells in vivo. HPCs and EPCs have been reported to express α5β1 and αLβ2, which have been shown to be important for the homing of these cells to the bone marrow and to sites of ischemia, respectively (13, 53).

As a therapeutic agent, THI0019 has advantages over other reported activators of integrins for stem cell therapy. First, it binds the target integrin directly and does not require any extensive preconditioning or genetic manipulation of cells. Second, THI0019 does not induce the binding of any of three different LIBS mAbs. This characteristic could be advantageous in the clinical setting because the induction of LIBS neoepitopes has been associated with acute thrombocytopenia in patients treated with αLβ3 integrin antagonists such as tirofiban and epifibatide (54). Third, the synergy seen with mAb TS2/16 allows for the potential of combination therapy to enhance the overall effect. Finally, large scale production of a low molecular weight small molecule such as THI0019 is likely to be less expensive than the production of previously reported biologic agents.

Acknowledgments—We thank Rebecca Bartow, Ph.D., and Nicole Stancel, Ph.D., of the Texas Heart Institute for editorial assistance and Deenadayalan Bakhavatsalam, Ph.D., for confocal microscopy advice. The Translational Chemistry Core Facility at the University of Texas M. D. Anderson Cancer Center was the recipient of National Institutes of Health Cancer Center Support Grant CA016672.

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