A Small Molecule That Inhibits the Interaction of Paxillin and \( \alpha 4 \) Integrin Inhibits Accumulation of Mononuclear Leukocytes at a Site of Inflammation*

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Extracellular antagonists of \( \alpha 4 \) integrin are an effective therapy for several autoimmune and inflammatory diseases; however, these agents that directly block ligand binding may exhibit mechanism-based toxicities. Inhibition of \( \alpha 4 \) integrin signaling by mutations of \( \alpha 4 \) that block paxillin binding inhibits inflammation while limiting mechanism-based toxicities. Here, we test a pharmacological approach by identifying small molecules that inhibit the \( \alpha 4 \) integrin-paxillin interaction. By screening a large (~40,000-compound) chemical library, we identified a nontoxic inhibitor of this interaction that impaired integrin \( \alpha 4 \)-mediated but not \( \alpha L \beta 2 \)-mediated Jurkat T cell migration. The identified compound had no effect on \( \alpha 4 \)-mediated migration in cells bearing the \( \alpha 4 \)(Y991A) mutation that disrupts the \( \alpha 4 \)-paxillin interaction, establishing the specificity of its action. Administration of this compound to mice led to impaired recruitment of mononuclear leukocytes to a site of inflammation in vivo, whereas an isomer that does not inhibit the \( \alpha 4 \)-paxillin interaction had no effect on \( \alpha 4 \)-mediated cell migration, cell spreading, or recruitment of leukocytes to an inflammatory site. Thus, a small molecule inhibitor that interferes with \( \alpha 4 \) integrin signaling reduces \( \alpha 4 \)-mediated T cell migration in vivo, thus providing proof of principle for inhibition of \( \alpha 4 \) integrin signaling as a target for the pharmacological reduction of inflammation.

Inhibition of \( \alpha 4 \) integrins is effective in alleviating a wide variety of chronic inflammatory diseases in animal models (1–4) by inhibition of the recruitment of leukocytes to sites of inflammation. In humans, anti-\( \alpha 4 \) antibodies, such as natalizumab, are of proven therapeutic effectiveness for the treatment of autoimmune diseases such as multiple sclerosis (5) or inflammatory bowel disease (6, 7). These antibodies and small molecule or peptidomimetic \( \alpha 4 \) integrin antagonists function by inhibiting the interaction of \( \alpha 4 \) integrins with ligands, such as vascular cell adhesion molecule-1. At full receptor blockade, these agents lead to complete loss of \( \alpha 4 \) function; hence, this approach carries the risk of serious side effects such as defects in placentation, heart development, and hematopoiesis (8).

Furthermore, the blockade of T cell entry into the central nervous system may account for the occurrence of progressive multifocal leukoencephalopathy in humans treated with anti-\( \alpha 4 \) integrin antibodies (9).

Integrins function as signaling molecules, and the tight binding of the adaptor protein paxillin to the cytoplasmic tail of the \( \alpha 4 \) integrin is necessary for certain \( \alpha 4 \)-mediated functions (10, 11). A mutation in the \( \alpha 4 \) cytoplasmic tail that selectively blocks the interaction of \( \alpha 4 \) integrin and paxillin (\( \alpha 4 \)(Y991A)) reduces T cell migration (12) as does inhibition of paxillin binding to \( \alpha 4 \) integrin by a fragment of paxillin (13). Importantly, blocking the \( \alpha 4 \)-paxillin interaction leaves \( \alpha 4 \) integrin-mediated static adhesion intact (10, 12), suggesting that interfering with \( \alpha 4 \) signaling is a more favorable therapeutic approach for the treatment of autoimmune diseases because it might obviate serious side effects seen with full blockade of the target.

Paxillin is the founding member of a protein family that consists of two other proteins, leupaxin (14) and Hic-5 (15). Leupaxin, which is homologous to paxillin and detectable as a 45-kDa protein, is preferentially expressed in hematopoietic cells (14). Paxillin and its parologue, leupaxin, contain multiple N-terminal leucine- and aspartic acid-rich sequences (LD domains) and LIM domains (16, 17) Both LD and LIM domains are important for protein-protein interactions, and the LIM domains mediate focal adhesion targeting of paxillin family members (18). Recent work also established a role for leupaxin in the function of osteoclasts (19) and in the migration of prostate cancer cells (20).

Transgenic mice that are homozygous for the \( \alpha 4 \)(Y991A) mutation are viable and fertile in stark contrast to the embryonic lethality seen in \( \alpha 4 \) integrin-null mice. Mice with disrupted \( \alpha 4 \)-paxillin interaction show defective recruitment of mononuclear leukocytes in thioglycollate-induced peritonitis; yet these mice have normal hemograms, normal abundance of hematopoietic precursors, and unimpaired homing of hematopoietic progenitor cells to the bone marrow (21). These findings lend further support to the idea that blockade of \( \alpha 4 \) integrin signaling can impair the recruitment of leukocytes to sites of inflammation while averting the adverse effects of complete loss of \( \alpha 4 \) integrin signaling.
inhibiting integrin function on development and hematopoiesis. Interfering with integrin signaling might therefore be a promising therapeutic approach for the treatment of chronic inflammatory diseases.

As noted above, there are now compelling genetic and cell biological data to suggest that blocking the α4-paxillin interaction could be therapeutically useful; however, to implement such a strategy, cell-permeable small molecule inhibitors of the interaction would be required. To this end, we previously screened a small combinatorial tripeptide library and found an antagonist, A7B7C7 (22). This compound was effective in inhibiting the α4-paxillin interaction in vitro (IC_{50} = 300 nM) and able to decrease α4β1-mediated T cell migration; however, later studies showed that this compound was cytotoxic in prolonged incubation with cells.

To test the feasibility of this strategy and to assess whether “classical” heterocyclic small molecules could block the α4-paxillin interaction, we screened a large (~40,000-compound) chemical library to identify small molecules that inhibit the α4-paxillin interaction. We identified a noncytotoxic inhibitor that impaired integrin α4-mediated but not α1β2-mediated Jurkat T cell migration. Furthermore, the α4-paxillin interaction inhibits cell spreading, and we found that the compound increased α4-mediated spreading. The specificity of the compound was verified by its lack of effect on the residual α4-mediated cell functions in cells bearing the α4(Y991A) mutation. As a direct test of the utility of this compound, we administered it to mice and found that it impaired the recruitment of leukocytes to a site of inflammation in vivo, whereas an isomer that does not inhibit the α4-paxillin interaction had no effect on α4-mediated cell migration, cell spreading, or recruitment of leukocytes to an inflammatory site.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Cell lines (Jurkat T cells, THP-1 cells and Chinese hamster ovary (CHO) cells) were obtained from American Type Culture Collection. Jurkat T cell lines expressing α4 with an alanine substitution at tyrosine 991 (JB4-Y991A cells) were generated as described before (12). Jurkat T cell lines were cultured in RPMI 1640 medium (Cellgro, Mediatech, Washington, D. C.) supplemented with 10% fetal bovine serum (Cellgro), 1% glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin (Sigma-Aldrich), and 1% nonessential amino acids (Invitrogen). THP-1 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 10 mM Heps, 50 μg/ml gentamycin, 50 μM 2-mercaptoethanol, and 50 μg/ml gentamycin. CHO cell lines expressing the α4 subunit or its mutant proteins have been described previously (23, 24). CHO cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin (Sigma-Aldrich), and 1% nonessential amino acids (Invitrogen).

**Reagents**—The mouse monoclonal horseradish peroxidase-conjugated antibody was purchased from BIOSOURCE (AM14404). The mouse monoclonal anti-hemagglutinin (HA) antibody (12CA5) was produced and purified in our laboratory (25). Purification of the human connecting segment-1 (CS-1) region of fibronectin fused to glutathione S-transferase (GST) has been described previously (26), using cDNA that was provided by J. W. Smith (Burnham Institute, La Jolla, CA). The cDNA for intracellular cell adhesion molecule (ICAM) was a generous gift from D. L. Simmons (CRF Laboratories, University of Oxford, Oxford, UK) and was modified as described before (27).

**Generation of Recombinant Proteins**—His_{6}-recombinant integrin cytoplasmic tail model proteins (α4 and αlβ2) were cloned into pET15b (Novagen) (11, 28) and modified by inserting a sequence encoding the peptide, GLNDIEAQKIEWHE, at the NdeI site immediately downstream of the thrombin cleavage site. This peptide directs incorporation of biotin in vivo upon expression in *Escherichia coli* by biotin ligase (Avidity, Denver) (29). Proteins were expressed and purified from *E. coli* extracts (28). Recombinant His_{6}-FAT, the focal adhesion targeting domain of pp125^{FAK}, was generated by PCR and cloned into pET15b (Novagen) between restriction sites BamHI and NdeI, expressed in BL21(DE3)pLysS cells (Novagen), and purified using His-bind resin (Novagen).

To generate recombinant full-length paxillin, leupaxin, or Git-1 with an N-terminal His_{6} tag and a C-terminal HA tag, oligonucleotides were generated and used in PCR to create cDNAs encoding the protein with an N-terminal HA tag. Each PCR product was cloned into pET28c (paxillin; Novagen) or pET15b (leupaxin, Hic-5, and Git-1; Novagen) between restriction sites BamHI and NdeI. Proteins were expressed in BL21(DE3)pLysS cells and purified using His-bind resin. Masses of proteins were assessed by SDS-PAGE and Coomasie staining.

**ELISA**—Thermo Electron High Binding 96 or 384 well plates were coated with 1 μg/ml neutravidin (Pierce) in 0.05 M NaHCO_{3}, pH 9.5, overnight at 4 °C. After blocking with 1% heat-inactivated BSA in PBS for 1 h at room temperature, plates were incubated with 1 μg/ml recombinant αlβ2-tail, α4-tail, FAT, or Git-1 in 1% BSA in PBS for 1 h at room temperature. Plates were washed twice with 0.2% Tween 20 in PBS to remove unbound protein and incubated with 10 μg/ml HA-tagged leupaxin or paxillin in 0.2% Tween 20, 1% BSA in PBS with or without 20 μg/ml recombinant α4 or FAT). Plates were washed twice, and bound leupaxin or paxillin was detected by incubating with the first antibody (12CA5 ascites, 1:10,000 in 0.2% Tween 20, 1% BSA in PBS) for 1 h at room temperature and then with the secondary antibody (horseradish peroxidase-conjugated anti-mouse (BIOSOURCE), 1:1,000 in 0.2% Tween 20, 1% BSA, PBS) for 1 h at room temperature. Binding was quantified after the addition of ECL solution on a Victor luminescence plate reader. 0% inhibition control was measured with leupaxin and no compound, and 100% inhibition control (background) was measured with no leupaxin and no compound. Each well was duplicated, and percent inhibition was averaged.

**Analysis of Inhibition of the α4-Leupaxin Interaction by 6-B345TTQ**—Inhibition of the α4-leupaxin interaction was measured using the above described ELISA. Biotinylated α4 integrin cytoplasmic tail was coated to the plates and incubated
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with increasing concentrations of leupaxin in the presence of different concentrations of inhibitor or no inhibitor. The data were fitted to equations for models of competitive, noncompetitive, uncompetitive, or mixed inhibition using GraphPad Prism 5.0 to test which model fits the best.

High Throughput Screening—Screening was performed using the above described ELISA that was translated into a 384-well plate format at the ICCB (Institute of Chemistry and Cell Biology) Longwood Screening Facility at Harvard Medical School, Boston, MA. The following libraries were screened: Mixed Commercials 1, 2, and 4, ChemDiv 1 and 2, Specplus, Maybridge 2 and 3, Peakdale2, Starr Foundation Extracts 1 and 2, Philippines Plant Extracts 2, DDS 2, Biomol ICCB known bio-reactives, Bionet 2, Enamine 1, and IF Lab 1.

Co-immunoprecipitation and Western Blotting—For endogenous co-immunoprecipitation experiments, Jurkat T cells were washed in ice-cold PBS and incubated with 1.6 mM Sulfo-NHS-Biotin (Pierce) for 30 min at room temperature. Excess biotin was quenched and washed from the cells with PBS and glycine. Cell lysates were then prepared with lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.05% Tween 20, and a protease inhibitor mixture (Roche Diagnostics Corp.). Lysate containing 200 μg of total cell protein was immunoprecipitated using 2 μg of RB-43S8 (rabbit anti-paxillin serum, generated in the laboratory). Immunoprecipitated proteins were separated by SDS-PAGE (8% BisTris, denaturing and reducing) and transferred to nitrocellulose membranes. Precipitated, biotinylated proteins were detected using horseradish peroxidase-streptavidin (1:10,000).

Migration Assay—Cell migration was assayed in a modified Boyden chamber assay system as described before (12). For Jurkat T cell migration, Transwell (Costar) polycarbonate membranes containing 3-μm pores were coated with 10 μg/ml CS-1, an α4 integrin-binding fragment of fibronectin, or 20 μg/ml ICAM in 0.1% NaHCO3, pH 8.0, overnight at 4 °C. Membranes were blocked with 2% BSA in PBS for 30 min at room temperature. 2.0 × 10^5 Jurkat T cells in RPMI 1640 medium with 10% FCS were added to the top chamber. Stromal-derived factor-1 (R&D Systems, Minneapolis, MN) at a final concentration of 15 ng/ml in RPMI 1640 medium with 10% FCS was added to the bottom chamber. Cells were allowed to migrate for 5 h at 37 °C. Cells in the bottom chamber were enumerated with a hemocytometer. Small molecule inhibitors were added to the top and bottom chambers in the indicated concentrations.

THP-1 cells were cultured in RPMI 1640 medium containing 0.1% fetal bovine serum for 16 h before 1 × 10^6 cells were added to the top chamber of Transwells with CS-1-coated membranes that contained 5-μm pores and allowed to migrate for 3 h toward RPMI 1640 medium containing 0.1% fetal bovine serum and 10 ng/ml recombinant mouse monocyte chemotactic protein-1 (R&D Systems).

Spreading Assay—Cell spreading was performed as described (10, 11). Briefly, CHO cells or Jurkat T cells resuspended in Dulbecco’s modified Eagle’s medium or RPMI 1640 medium, respectively, containing 0.5% FCS and 0.2% BSA, were plated on coverslips coated with 3 μg/ml GST-CS-1 in 12-well plates in the presence or absence of the small molecule inhibitor. After spreading for 45 or 180 min at 37 °C, respectively, cells were fixed, permeabilized, and stained with rhodamine-conjugated phalloidin. The cell area was measured and analyzed using ImageJ software. At least three independent experiments were performed, and in each experiment at least 30 cells were analyzed.

Cytotoxicity Assay—The cytotoxicity effect of compounds on cultivated cells was determined using the CellTiter 96® Aqueous One Solution cell proliferation assay (Promega, Madison, WI) according to the manufacturer’s guidelines.

Thioglycollate-induced Peritonitis—Male C57BL/6 mice 5–6 weeks old were injected intraperitoneally with 1 ml of sterile 4% (w/v) thioglycollate (Sigma-Aldrich). Mice were injected with 16.5 mg/kg 6-B345TTQ, 6-B234TTQ, or the equal volume of vehicle every 8–12 h. At various time points after injection, mice were killed and subjected to peritoneal lavage with 4 ml of PBS containing 5 mM EDTA and 1% BSA. Total leukocytes in lavage samples were enumerated with a hemocytometer. Cells (1 × 10^5) were attached to glass slides with a Cytospin 4 instrument (ThermoShandon) and stained with modified Wright-Giemsa stain. Differential cell counts were performed on five individual fields, each containing 100 cells by light microscopy.

Hematology—Peripheral blood from the tail vein was collected in tubes containing EDTA. Complete blood counts and leukocyte differentials were performed using a Hemavet 850FS Multispecies hematology system (Drew Scientific, Waterbury, CT).

RESULTS

High Throughput Screening—To identify small molecule inhibitors for the α4 integrin-leupaxin/paxillin interaction we used an ELISA and the ICCB Longwood screening facilities at Harvard Medical School. His6 recombinant integrin cytosplastic tail model proteins (α4 and αiib) were cloned (11, 28) and modified by inserting a sequence encoding the peptide, GLN-DFEAKQIEWHE, at the NdeI site immediately downstream of the thrombin cleavage site. This peptide incorporates biotin in vivo upon expression in E. coli by biotin ligase. Proteins were expressed and purified from E. coli extracts (28). A total of 40,000 compounds was screened at a concentration of 16 μg/ml using the Mixed Commercials 1, 2, and 4, ChemDiv 1 and 2, Specplus, Maybridge 2 and 3, Peakdale2, Starr Foundation Extracts 1 and 2, Philippines Plant Extracts 2, DDS 2, Biomol ICCB known bio-reactives, Bionet 2, Enamine 1, and IF Lab 1 libraries. As a positive control for inhibition, we used compound A7B7C7 (22) at a final concentration of 25 μM. Compounds that showed inhibition of at least 60% were considered screening positives. A total of 194 compounds (~0.5% of the compounds screened) met this criterion. 0.3% (115 compounds) were then retested in the ELISA at different concentrations. 25 (0.06%) of the total compounds screened were confirmed as screening positives.

Compound 6-B345TTQ Inhibits the Interaction of α4-Integrin with Leupaxin Acting as a Competitive Inhibitor—In dose-response binding ELISAs, we verified the inhibitory effect of compound 6-B345TTQ (ChemDiv, San Diego, CA) on the interaction of leupaxin with α4 integrin. Interestingly, an iso-
Inhibiting the α4-leupaxin interaction. Compared with A7B7C7, which inhibited the interaction of leupaxin with the FAT sequence of pp125FAK with an IC\textsubscript{50} of 300 nM, 6-B345TTQ had no effect on the binding of FAT to leupaxin at concentrations up to 100 μM (Fig. 2C). Interestingly, it did inhibit the interaction of FAT with paxillin. Comparing the effect of 6-B345TTQ on the interaction of α4 integrin with different paxillin paralogues, we found that leupaxin and paxillin binding were inhibited with IC\textsubscript{50} values of 18 μM or 10.8 μM, respectively (Fig. 2C). To investigate further the characteristics of the inhibitor, we tested its effect on the interaction of leupaxin or paxillin with the Arf-GAP, Git-1, which binds to the LD4 motif of paxillin (30, 31). Compound 6-B345TTQ had a strong inhibitory effect on the interaction of GIT-1 with paxillin (IC\textsubscript{50} = 2.4 μM) and a weaker effect on its interaction with leupaxin (IC\textsubscript{50} = 25 μM) (Fig. 2B). In contrast, the inactive isomer 6-B234TTQ did not inhibit the interaction of paxillin with either FAT or Git-1. The previously identified compound A7B7C7 inhibited the interaction of paxillin with both FAT and Git-1 (IC\textsubscript{50} = 1.8 and 1.7 μM, respectively) (Fig. 2D).

To investigate the nature of inhibition by 6-B345TTQ, α4 was immobilized on ELISA plates and incubated with increasing concentrations of leupaxin in the presence or absence of various concentrations of control compound 6-B234TTQ or A7B7C7. Error bars, S.E.

These results indicate that 6-B345TTQ acts as a competitive inhibitor.
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To confirm the effect of compound 6-B345TTQ on the interaction of native paxillin and α4 integrin, paxillin was immunoprecipitated from surface biotin-labeled Jurkat T cells in the presence of increasing concentrations of compound 6-B345TTQ or the control compound 6-B234TTQ (n.c.). A co-immunoprecipitated surface polypeptide of this mobility was previously identified as the α4 integrin subunit (10). Immunoprecipitated paxillin was detected by anti-paxillin antibody.

Compound 6-B345TTQ Inhibits α4-Mediated Cell Migration of T Cell and Monocyte Cell Lines and Enhances Cell Spreading—The ability of α4 to enhance cell migration is dependent on the binding of paxillin (10, 11), leading us to investigate the effect of compound 6-B345TTQ on Jurkat T cell or THP-1 monocyte migration. Directed α4-mediated migration was assessed in a modified Boyden chamber using fibronectin CS-1 fragment (α4 integrin derived from cells, we immunoprecipitated paxillin from Jurkat T cells in the absence or presence of the compound (Fig. 4). α4 Integrin was present in paxillin immunoprecipitates, and the amount of co-immunoprecipitated α4 decreased with increasing concentrations of 6-B345TTQ. The control compound 6-B234TTQ did not reduce the amount of co-precipitated α4.

FIGURE 3. 6-B345TTQ acts as a competitive inhibitor. Biotinylated recombinant α4 tails were immobilized on neutravidin-coated 96-well plates. Bound proteins were incubated with increasing concentrations of HA-tagged recombinant leupaxin in the presence or absence of compound 6-B345TTQ. Bound leupaxin was detected by incubation with an anti-HA tag antibody followed by horseradish peroxidase conjugate and detected by luminiscence. Data were fitted to equations for models of competitive, noncompetitive, uncompetitive, or mixed inhibition using GraphPad Prism 5.0. Error bars, S.E.

FIGURE 4. Compound 6-B345TTQ impairs the interaction of native paxillin with α4 integrin. Paxillin was immunoprecipitated from surface biotin-labeled Jurkat T cells in the presence of increasing concentrations of compound 6-B345TTQ or the control compound 6-B234TTQ (n.c.). A co-immunoprecipitated surface polypeptide of this mobility was previously identified as the α4 integrin subunit (10). Immunoprecipitated paxillin was detected by anti-paxillin antibody.

FIGURE 5. Incubation of monocytes or Jurkat T cells with compound 6-B345TTQ leads to decreased α4-mediated cell migration. Effect of 6-B345TTQ on α4- or αLβ2-mediated Jurkat T cell or on α4-mediated monocyte (THP-1) migration is shown. Cell migration was assayed in a modified Boyden chamber assay. Transwell polycarbonate membranes were coated with 10 μg/ml CS-1 or 20 μg/ml ICAM. 1.0 × 105 THP-1 cells or 2.0 × 105 Jurkat T cells were added to the top chamber and allowed to migrate for 3 or 5 h, toward monocyte chemotactic protein-1 or stromal-derived factor-1α, respectively. Cells in the bottom chamber were enumerated using a hemocytometer. Small molecule inhibitors were added to the top and bottom chambers at the indicated concentrations. n.c., negative control compound 6-B234TTQ. Results are mean ± S.E. (error bars) of five independent experiments. *, p < 0.05 compared with 0 μm compound, two-tailed Student’s t test.

FIGURE 6. Compound 6-B345TTQ has no effect on cell migration of Jurkat T cells expressing an α4(Y991A) mutant that fails to bind paxillin. Cell migration was assayed in a modified Boyden chamber assay. Transwell polycarbonate membranes containing 3-μm pores were coated with 10 μg/ml CS-1 or 20 μg/ml ICAM. 2.0 × 105 Jurkat T cells expressing wild-type α4 (Jurkat) or α4(Y991A) (JB4-Y991A) in RPMI 1640 with 10% FCS were added to the top chamber and allowed to migrate for 5 h at 37 °C toward stromal-derived factor-1α. Cells in the bottom chamber were enumerated with a hemocytometer. Small molecule inhibitors were added to the top and bottom chambers in the indicated concentrations. Results are mean ± S.E. (error bars) of five independent experiments. *, p < 0.05 compared with 0 μm compound, two-tailed Student’s t test.

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that the compound specifically inhibits α4-mediated Jurkat T cell migration.

As an additional test for toxicity of this compound, we examined its effects on cell viability; the compound was not cytotoxic at concentrations up to 100 μM for 24 h. In sharp contrast, incubation with 50 μM A7B7C7 for 24 h led to 62% dead cells after 24 h of incubation (Fig. 7).

Expression of a phosphorylation-mimicking α4 variant in CHO cells (CHO-α4S998D cells) leads to reduced cell migration and enhanced cell spreading due to the disruption of paxillin binding to α4 (24). In contrast, CHO cells that express the nonphosphorylatable mutant (CHO-α4S998A cells) have a spreading defect (23, 24) due to the recruitment of Arf-GAPs such as Git-1 (32). Therefore, we used the spreading defect of CHO-S998A cells as a convenient readout for biological consequences of inhibition of paxillin binding to α4 integrin by 6-B345TTQ. CHO-S998A cells were plated on CS-1-coated coverslips and were allowed to spread for 45 min in the presence of increasing concentrations of 6-B345TTQ. Incubation with the compound increased the spreading of CHO-S998A cells to a level comparable with CHO-S998D cells, whereas the control compound 6-B234TTQ had no effect (Fig. 8B). The same result was observed in Jurkat T cells when spreading was assessed 180 min after plating on CS-1. In presence of increasing concentrations of the compound, a dose-dependent increase of cell spreading was observed, reaching a level comparable with that of cells (JB4-Y991A) that express an α4 mutant with impaired paxillin binding (Fig. 8A). In contrast, compound 6-B345TTQ did not increase cell spreading of CHO cells that adhere to fibronectin via α5β1 integrin (data not shown).

6-B345TTQ Impairs Recruitment of Mononuclear Leukocytes to an Inflammatory Site—To test directly the effect of compound 6-B345TTQ on leukocyte recruitment in vivo, C57BL/6 mice were injected intraperitoneally with thioglycollate to induce inflammation. Mice were injected with 16.5 mg/kg 6-B345TTQ or 6-B234TTQ (control compound) or the equal volume of vehicle every 8–12 h. Mice that were injected with vehicle showed a 10.5-fold increase in peritoneal monocytes/macrophages 48 h after thioglycollate injection. In mice that were treated with 6-B345TTQ, infiltration of monocytes/macrophages was reduced by 50% (Fig. 9A). The recruitment of lymphocytes to the peritoneum was also reduced 48 h and 72 h after thioglycollate injection in mice that were injected with 6-B345TTQ compared with control mice, but this reduction was not statistically significant. There was no difference in the recruitment of neutrophils in response to thioglycollate between mice that were injected with the compound or with vehicle only, showing that the compound did not block host recognition of the thioglycollate. Furthermore, the control compound 6-B234TTQ had no effect on leukocyte migration into the inflamed peritoneum (data not shown). To verify that impaired migration of leukocytes to the inflamed peritoneum was not due to leukopenia, peripheral blood counts were taken from mice before and 48 h after injection of the compound. The number of circulating leukocytes/μl of blood (8,400 ± 2,100, n = 3 (mean ± S.D.)) 48 h after compound treatment was not reduced relative to pretreatment values (6,600 ± 1,300, n = 3). Similarly, 48 h after compound injection, the number of monocytes (520 ± 200 versus 350 ± 50, n = 3) or lymphocytes (6,100 ± 1,600 versus 4,900 ± 1,000, n = 3) increased relative to preinjection values.
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**FIGURE 9.** Recruitment of mononuclear leukocytes to the peritoneum in response to thioglycollate is impaired in C57BL/6 mice treated with 6-B345TTQ. C57BL/6 mice were injected intraperitoneally with 1 ml of sterile 4% (v/v) thioglycollate. Mice were treated with 16.5 mg/kg 6-B345TTQ or the equal volume of vehicle every 8 – 12 h by intraperitoneal injection. At various time points after injection, mice were killed and subjected to peritoneal lavage with 4 ml of PBS containing 5 mM EDTA and 1% BSA. Total leukocytes in the lavage samples were enumerated with a hemocytometer, and differential cell counts were performed on cytoospin slides after modified Wright-Giemsa staining. Results are shown for total monocyte/macrophage (A), lymphocyte (B), and neutrophil (C) counts. *, p < 0.05, two-tailed Student’s t test. Results are mean ± S.E. (error bars) of 10 mice from three separate experiments.

**DISCUSSION**

Because of the important role of α4 integrins in inflammatory processes, they have become targets for the treatment of chronic inflammatory disorders. Although anti-α4 antibodies or small molecule antagonists that block ligand binding are effective, this approach is limited by mechanism-based toxicities. We have screened several chemical libraries and identified and characterized a small molecule inhibitor (6-B345TTQ) that disrupts the interaction of paxillin and α4 integrin and thereby interferes with α4 integrin signaling. Transgenic mice that are homozygous for a mutation in the α4 integrin cytoplasmic tail (α4Y991A) that leads to the disruption of paxillin binding (12, 21) are viable, and their viability and fertility stand in stark contrast to the embryonic lethal α4 integrin-null mice (33). Mice with a disrupted α4-paxillin interaction show defective recruitment of mononuclear leukocytes in thioglycollate-induced peritonitis while having normal hemograms, normal abundance of hematopoietic precursors, and unimpaired homing of hematopoietic progenitor cells to the bone marrow (21), suggesting that inhibition of the α4-paxillin interaction could be of use in blocking leukocyte recruitment to inflammatory sites. The present findings show that pharmacological blockade of the α4 integrin-paxillin interaction impairs α4β1-dependent migration and the recruitment of leukocytes, thus providing proof of principle for this novel antiinflammatory strategy.

Here, we report on a small molecule (6-B345TTQ) that inhibits the α4-paxillin interaction in cells in vitro by acting as a competitive inhibitor. A compound with an identical structure except for one methoxy group that is shifted from position 5 to 2 had no inhibitory effect on this interaction and was used as a control. Compound 6-B345TTQ inhibited α4-mediated monocyte and T cell migration by 52.6% and 56.4%, respectively, but did not significantly reduce T cell migration mediated by other α integrin subunits, suggesting a specific inhibitory effect on α4-mediated cell migration. Furthermore, it failed to block the residual migration of cells bearing the α4(Y991A) mutation, providing definitive proof that the compound blocks migration by inhibiting the α4-paxillin interaction. It will be of interest for future work to examine the effect of this compound on firm adhesion under fluid shear stress.

This compound exhibited notable specificity with respect to the inhibition of interactions of paxillin paralogues in vitro. 6-B345TTQ blocked the binding of leupaxin and paxillin to α4; hence, its antimigratory effects are likely to be independent of which paxillin paralogues are expressed. It had no effect on interactions of leupaxin with FAK or with Git-1, interactions that are important in the signaling downstream of integrins (34). In sharp contrast, compound A7B7C7 (22) inhibited these interactions, establishing the increased specificity of this compound. Furthermore, because FAK signaling plays an important role in cell survival (35), this differing specificity could explain the differential effects of A7B7C7 and 6-B345TTQ on cell death.

Surprisingly, 6-B345TTQ inhibited the interaction of paxillin with FAT and Git-1. The α4 integrin binding site in paxillin has been localized to a 100-amino acid stretch encompassing LD3 and LD4 (13). FAK is known to bind to LD2 and LD4 of paxillin, but the exact binding site in leupaxin and Hic-5 has not been identified yet (16, 17, 36, 37). Leupaxin and Hic-5 both lack one LD motif, and their LD3 motif is structurally similar to LD4 of paxillin. The structure of LD2 of Hic-5 and paxillin is similar but is lacking in leupaxin (14, 15). These sequence differences might account for the different effect of compound 6-B345TTQ on the binding of FAT to paxillin compared with leupaxin.

In thioglycollate-induced peritonitis, administration of the compound resulted in significantly reduced infiltration of monocytes/macrophages 48 h after induction of inflammation. A similar effect was observed in transgenic α4(Y991A) mice expressing a mutated α4 integrin that leads to disruption of paxillin binding (21). Additionally, neutrophils in both transgenic α4(Y991A) mice and C57BL/6 mice treated with compound 6-B345TTQ were not affected. Recruitment of lymphocytes to the inflamed peritoneum was also reduced in both C57BL/6 mice that were treated with compound 6-B345TTQ and α4(Y991A) mice, although the reduction in compound-treated mice was not statistically significant. Interestingly, the negative control compound 6-B234TTQ had no discernible effect on the recruitment of leukocytes in vivo.

These data add weight to the proposal that integrin signaling is a potential therapeutic target. In particular, we describe a small molecule inhibitor that interferes with α4 binding to a specific intracellular target and thus reduces α4-mediated leukocyte recruitment in vivo.
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