

Technical Advance: Function and efficacy of an α_4 -integrin antagonist using bioluminescence imaging to detect leukocyte trafficking in murine experimental colitis

Carola T. Murphy,* Gerard Moloney,*[†] John MacSharry,* Andrea Haynes,[†] Emilie Faivre,* Aoife Quinlan,* Peter G. McLean,[†] Kevin Lee,[†] Liam O'Mahony,* Fergus Shanahan,*¹ Silvia Melgar,*[†] and Kenneth Nally*

*Alimentary Pharmabiotic Centre, University College Cork, National University of Ireland, Cork, Ireland; and [†]Immunology, CEDD, GlaxoSmithKline, Stevenage, United Kingdom

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ABSTRACT

Leukocyte trafficking is a therapeutic target in IBD. The integrins $\alpha_4\beta_7$ and $\alpha_4\beta_1$ regulate leukocyte migration into tissues and lymphoid organs. Current strategies rely on biologics, such as mAb, to inhibit leukocyte recruitment. Here we show the in vivo therapeutic effects of a small molecule α_4 -integrin antagonist (GSK223618A) in a leukocyte-trafficking model and a murine model of colitis. Leukocytes isolated from MLNs of transgenic β -actin-*luc*⁺ mice were injected i.v. into recipients with DSS-induced colitis. Recipient mice were orally gavaged with vehicle or an α_4 -integrin antagonist 1 h pre-adoptive transfer, followed by bioluminescence whole body and ex vivo organ imaging 4 h post-transfer. To confirm its therapeutic effect, the α_4 -integrin antagonist was given orally twice daily for 6 days to mice with DSS-induced colitis, starting on Day 3. Clinical, macroscopic, and histological signs of inflammation were assessed and gene-expression profiles analyzed. Using bioluminescence imaging, we tracked and quantified leukocyte migration to the inflamed gut and demonstrated its inhibition by a small molecule α_4 -integrin antagonist. Additionally, the therapeutic effect of the antagonist was confirmed in DSS-induced colitis in terms of clinical, macroscopic, and histological signs of inflammation.

Abbreviations: CD=Crohn's disease, C_{max}=maximum plasma concentration, Ct=comparative threshold, DDAI=daily disease activity index, DSS=dextran sodium sulfate, Foxp3=forkhead box p3, GATA3=GATA binding protein 3, Hsp=heat shock protein, IBD=inflammatory bowel disease, ITG α_4 =integrin α_4 , *luc*⁺=luciferase-expressing, MLN=mesenteric lymph node, MS=mass spectrometry, PK=pharmacokinetic, p.o.=per os (by mouth), PP=Peyer's patches, PTGS2=PG synthase 2, qRT-PCR=quantitative RT-PCR, ROI=region of interest, ROR=retinoic acid receptor-related orphan receptor, T-bet=T-box expressed in T cells, UC=ulcerative colitis

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Gene expression analysis suggested enhancement of tissue healing in compound-treated animals. Inhibition of leukocyte trafficking using small molecule integrin antagonists is a promising alternative to large molecule biologics. Furthermore, in vivo bioluminescence imaging is a valuable strategy for preclinical evaluation of potential therapeutics that target leukocyte trafficking in inflammatory diseases. *J. Leukoc. Biol.* **88**: 1271–1278; 2010.

INTRODUCTION

Leukocyte recruitment to sites of inflammation is a multistep process involving sequential engagement of selectins, chemokines, integrins, and their counter endothelial cell ligands [1]. The integrins $\alpha_4\beta_7$ and $\alpha_4\beta_1$ have an essential regulatory role in leukocyte homing and recruitment to inflammatory tissues. Expression of the α_4 -integrin ligands, mucosal addressin-cell adhesion molecule 1 (MAdCAM-1) and vascular-cell adhesion molecule 1 (VCAM-1), is up-regulated in the inflamed colonic mucosa of CD and UC patients and in animal models of IBD [2, 3]. Therefore, inhibition of leukocyte trafficking is currently being exploited as a therapeutic target for IBD.

Promising preclinical studies paved the way for use of humanized α_4 -integrin antibodies in human clinical trials [4–6]. Natalizumab (Tysabri) is a humanized pan- α_4 mAb that blocks binding of $\alpha_4\beta_7$ and $\alpha_4\beta_1$ to their respective ligands on the endothelium, thus inhibiting lymphocyte transendothelial migration [7]. It has proven effective in treating multiple sclerosis [8, 9] and in preventing relapse and increasing remission rates in sufferers with moderate to severe CD [7, 10]. MLN-02

1. Correspondence: Alimentary Pharmabiotic Centre, University College Cork, National University of Ireland, Cork, Ireland. E-mail: f.shanahan@ucc.ie

is a humanized IgG1 mAb selective for $\alpha_4\beta_7$ and has been reported to induce remission in UC [11]. However, undesirable immunogenic responses associated with such protein therapy [7, 8] highlight the attractiveness of using non-peptide small molecule integrin antagonists to block leukocyte recruitment and treat inflammatory diseases.

Bioluminescence imaging is based on the detection of visible light photons, produced during the enzymatic oxidation of luciferin by luciferase, when the enzyme is expressed *in vivo* as a molecular reporter [12]. Firefly luciferase has a broad-band emission wavelength that peaks at 562 nm, thus facilitating light penetration through several centimeters of biological tissue and providing organ-level resolution [13]. In contrast to fluorescent reporters, such as GFP, luciferase proteins do not require external excitation, and no background signal is generated from the reporter. In addition, this technique is non-invasive and allows direct visualization of molecular processes within the required setting, during short time periods.

In this study, we used *in vivo* bioluminescence imaging to investigate the functional blockade effect of a small molecule α_4 -integrin antagonist, GSK223618A [14, 15], on leukocyte trafficking to the inflamed gastrointestinal tract. We further investigated the therapeutic potential of this compound in a chemically induced DSS murine model of colitis. These results demonstrate that the small molecule α_4 -integrin antagonist is effective at inhibiting leukocyte trafficking *in vivo* and at ameliorating disease during colitis. Moreover, they highlight the use of bioluminescence imaging as a tool for testing potential therapeutics that block leukocyte recruitment in autoimmune diseases.

MATERIALS AND METHODS

Mice

For the PK study, three male C57BL/6J mice were obtained from Charles River (UK). For the bioluminescence imaging leukocyte trafficking study, male WT FVB/N mice, 8–12 weeks old, were obtained from Harlan (UK). The β -actin/*luc*⁺ transgenic FVB/N mice carry a 14-kb fragment of the murine β -actin promoter isolated from genomic DNA, a chimeric intron, and modified firefly luciferase cDNA (Promega pGL-3) and were purchased from Caliper Life Sciences (Alameda, CA, USA). For the therapeutic efficacy study in DSS experimental colitis, specific pathogen-free female C57BL/6OlaHsd mice, 7–12 weeks old, weighing 17–20 g, were obtained from Harlan. All mice were housed in a conventional environment (temperature 21°C, 12 h light:12 h darkness, humidity 50%) in a dedicated animal holding facility. They were fed a standard non-sterile pellet diet and tap water *ad libitum*. Mice were allowed ≥ 2 weeks to acclimatize before entering the study. Animal husbandry and experimental procedures were approved by the University College Cork Animal Ethics Committee (Ireland).

PK study

The PK activity of the α_4 -integrin antagonist, GSK223618A [14, 15], was investigated in three male C57BL/6J mice. The compound was suspended in 1% (w/v) methylcellulose (Sigma-Aldrich Co. Ltd., Dorset, UK) at a concentration of 2 mg/ml and orally administered at 10 mg/kg. Serial blood samples were taken from the tail-vein of each mouse up to 12 h after dosing. Diluted blood samples were analyzed for

the drug by liquid chromatography/MS/MS (lower limit of quantitation was 2 ng/mL, 0.004 μ M).

DSS-induced colitis and treatment with α_4 -integrin antagonist

For the leukocyte trafficking study using bioluminescence imaging, colitis was chemically induced in the recipient mice using DSS as described previously [16]. Briefly, individually housed FVB/N mice were given 4% filtered DSS (47 kDa; TdB Consultancy, Uppsala, Sweden) *ad libitum* in their drinking water for 5 days followed by water for the remainder of the study. DSS was changed once during the 5 days. DSS recipient mice were orally gavaged with vehicle or the α_4 -integrin antagonist at 30 mg/kg (p.o.) 1 h pre-adoptive transfer of the *luc*⁺ donor leukocytes. Of note, we and others [17] have shown the FVB/N strain to be more resistant to DSS-induced colitis compared with the C57BL/6 strain. Thus, a higher dose of DSS and a different duration of administration were required for the bioluminescence imaging studies.

To assess therapeutic efficacy of the α_4 -integrin antagonist, DSS was added to the drinking water (normal tap water) of C57BL/6 mice to a final concentration of 3% w/v and prepared freshly every day. All groups were administered DSS for 6 days, followed by 3 days on tap water, and killed for sample and tissue processing on Day 9. The mice were randomized into four groups of eight, and each group was orally gavaged with vehicle (1% methylcellulose, Sigma-Aldrich Co. Ltd.) or the test compound at 10 mg/kg, 30 mg/kg, or 90 mg/kg p.o. twice daily from Day 3 until Day 8 of the DSS trial. Non-DSS (control) mice were also randomized in four groups of five mice and gavaged with vehicle and three concentrations of the test compound, according to the same schedule as the DSS-treated mice.

Isolation of *luc*⁺ donor leukocytes for bioluminescence imaging leukocyte trafficking study

The luciferase reporter gene is expressed, and luciferase activity is detectable in all tissue, including white blood cells of the transgenic β -actin-*luc*⁺ mice [18]. It has been demonstrated previously that *luc*⁺ cells emit visible light photons that penetrate tissues and are detectable externally and quantitatively with high sensitivity [19, 20]. Thus, *luc*⁺ cells were isolated for use in the leukocyte trafficking study. Briefly, MLNs were harvested from healthy transgenic β -actin-*luc*⁺ mice and placed in 5 ml RPMI-1640 media (Gibco, UK), supplemented with 10% heat-inactivated FCS (Gibco) and 1% Pen/Strep (Gibco). A 50 μ m sterile Medicon (BD Biosciences, UK) was used to release the cells from the lymph nodes and remove any fatty tissue. The lymph node cells were filtered through a 70- μ m nylon cell strainer (BD Falcon, UK). Cells were kept on ice throughout processing. Cells were washed by centrifuging at 300 *g* for 10 min at 4°C, pooled, and counted using the Trypan blue exclusion method.

In vivo bioluminescence imaging

Following 5 days of DSS exposure and 1 day of water, 10 million *luc*⁺ leukocytes were adoptively transferred i.v. into the tail-vein of FVB/N recipients with DSS-induced colitis. Four hours postadoptive cell transfer, *in vivo* bioluminescence imaging was performed, as described previously [19, 21], using an IVIS 100 charge-coupled device imaging system (Xenogen, Alameda, CA, USA). Briefly, the recipient mice were injected i.p. with the exogenous substrate D-luciferin (120 mg/kg body weight; BioThema AB, Sweden), following gaseous anesthesia with 3% isoflurane, and transferred to the imaging chamber. Emission images were collected with 2 min integration times. Following the whole body imaging at the 4-h time-point, the mice were injected with an additional dose of D-luciferin. Five minutes later, the mice were killed via cervical dislocation, and organs were removed and imaged for 2 min. Bioluminescence signal was quantified by

creation of ROIs. To standardize the data, light emission was quantified from the same surface area (ROI) for each organ type. In addition, background light emission, from ROIs created on organs of non-recipient, non-DSS control animals, was subtracted from test organs. Imaging data were analyzed and quantified with Living Image software (Xenogen) and expressed as photons/s/cm².

Clinical and macroscopic assessment of inflammation

For the efficacy study during experimental colitis, body weight, stool consistency (0=normal, well-formed pellets; 1=changed-formed pellets; 2=loose stool; 3=diarrhea), and fur texture/posture (0=smooth coat/not hunched; 1=mildly scruffy/mildly hunched; 2=very scruffy/very hunched) were recorded to generate a DDAI. Colons were removed from anus to cecum, opened longitudinally, washed in PBS, and separated into proximal and distal regions. The length and weight of each region were recorded along with a macroscopic inflammatory score, based on the degree of inflammation (0–4), presence of edema (0–3), stiffness (0–2), and/or ulcerations (0–1), resulting in a total macroscopic score of 10 [22]. Scoring of inflammatory parameters was performed in a blinded manner. The distal colon (3 cm) was cut longitudinally into two sections. One section was rolled and frozen in Jung tissue-freezing medium (Leica Instruments, Nussloch, Germany) and liquid N₂ and stored at –80°C for histological analysis. The other distal colon section was snap-frozen and stored at –80°C for qRT-PCR analysis.

Histology

Frozen colonic sections (5–7 μ m) were stained with H&E, according to standard histological procedures. The sections were coded and observed with a light microscope (Olympus BX51, Olympus, Germany) in a blinded manner (S. Melgar). A histology (microscopic) score, reflecting infiltration of inflammatory cells and epithelial structure, was used on a scale of 0–6, where 0 = no signs of damage; 1 = few inflammatory cells, no signs of epithelial degeneration; 2 = mild inflammation, few signs of epithelial degeneration; 3 = moderate inflammation, few epithelial ulcerations; 4 = moderate to severe inflammation, ulcerations in >25% of the tissue section; 5 = moderate to severe inflammation, large ulcerations of >50% of the tissue section; 6 = severe inflammation and ulcerations of >75% of the tissue section [23]. The extent of ulcerated and nonulcerated areas was measured on the whole tissue section at 10 $\times$ magnification using the Cell F software (Olympus) and was expressed as percent ulcer extent of total tissue. These measurements were only performed in the vehicle and 30 mg/kg dose-treated groups exposed to DSS.

Real-time qRT-PCR gene expression analysis

Colonic mucosal mRNA expression was evaluated using qRT-PCR. Total RNA was isolated from colonic tissue using the Absolutely RNA RT-PCR Miniprep kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's protocol. cDNA was synthesized using 1 μ g total RNA. PCR primers and probes were designed using the Universal ProbeLibrary Assay Design Center (<https://www.roche-applied-science.com/sis/rtqpcr/upl/adc.jsp>; Roche Applied Science, Indianapolis, IN, USA). Primer sequences are available upon request. β -actin and hexose-6-phosphate dehydrogenase were used as housekeeping genes to correct for variability in the initial amount of total RNA. All PCR reactions were performed in triplicate using 384-well plates on the LightCycler 480 system (Roche Applied Science). Positive and negative controls were also included. The $2^{-\Delta\Delta C_t}$ method [24] was used to calculate relative changes in gene expression determined from real-time qRT-PCR experiments. The following genes were analyzed: *IFN- γ* , *IL-1 β* , *IL-6*, *IL-8*, *IL-10*, *IL-11*, *IL-12*, *IL-17*, *IL-22*, *TNF- α* , *T-bet*, *ROR γ* , *Foxp3*, *GATA-3*, *NOS2/iNOS*, *ITG α_4* , *VEGFA*, *Hsp70/Hspa1b*, *PTGS2/COX-2*, *CCL-2*, *CCL-11*, *CCL-20*, and *CXCL10*.

Statistical analysis

Statistical significance was determined with one-way ANOVA with post-hoc analysis and the unpaired Student's *t* test. qRT-PCR expression data, following $2^{-\Delta\Delta C_t}$ analysis, were subjected to the Mann-Whitney *U* test to compare all groups. All statistical tests were performed using commercially available statistic software (GraphPad Software, San Diego, CA, USA). Data are represented by mean $\pm$ SEM. *P* values of <0.05 were considered significant.

RESULTS

Adoptively transferred leukocytes were efficiently tracked and quantified by bioluminescence imaging

To establish an efficient model to track and quantify leukocyte migration, we developed an *in vivo* leukocyte-trafficking model using a *luc*⁺ transgenic donor mouse line in conjunction with bioluminescence imaging. Four hours after transfer of *luc*⁺ cells, a clear bioluminescence signal was apparent in the whole body and in *ex vivo* organ images of all of the recipient DSS mice in contrast to the non-recipient, non-DSS control. Specifically, in whole body images, bioluminescence signals were observed in regions indicative of leukocyte homing to cervical lymph nodes, lungs, spleen, and the lower abdominal area (Fig. 1A, and data not shown). Imaging of isolated organs confirmed migration of cells to these areas as well as to the MLNs, Peyer's patches (PPs), and large intestine.

A small molecule α_4 -integrin antagonist reduced leukocyte trafficking to the distal colon and small intestine of DSS-colitic mice

The small molecule α_4 -integrin antagonist was orally gavaged at 10 mg/kg to C57BL/6 mice and displayed consistent systemic exposure with a mean dose normalized area under the plasma concentration/time curve of 48 ± 5 min/kg/L. The absorption of the compound was rapid (*C*_{max} of 6.14 ± 0.64 μ M, achieved in 1.0 h), and the time after administration of a drug when the *C*_{max} of the compound is reached was 1 h (range 0.5–1.0 h; data not shown). Based on the PK data, the compound (30 mg/kg) was orally gavaged 1 h preadoptive transfer of the *luc*⁺ cells, and the mice were imaged 4 h thereafter. Whole body imaging showed a significantly lower bioluminescence signal from the lower abdominal area of the DSS recipient mice that received the α_4 -integrin antagonist compared with those that received the vehicle alone (Fig. 1B). *Ex vivo* imaging of the organs postmortem revealed a high bioluminescence signal in the lungs and spleens of the vehicle-treated and α_4 -integrin antagonist-treated DSS recipients, and lower signals were apparent in the livers of both groups. Localization of leukocytes to these systemic organs occurs, as donor cells were administered to recipient animals via the tail-vein and consequently, followed the natural route of circulation. In agreement with this, we have also observed distribution of adoptively transferred cells to lungs, liver, and spleens of naïve mice (data not shown). Furthermore, there was no significant increase or decrease in signal from lungs and spleens in the compound-treated group compared with vehicle-treated group, suggesting that the α_4 -integrin antagonist had no effect on cell trafficking to these areas (Fig. 1E). However, bioluminescence imaging revealed a significant reduction in signal from the

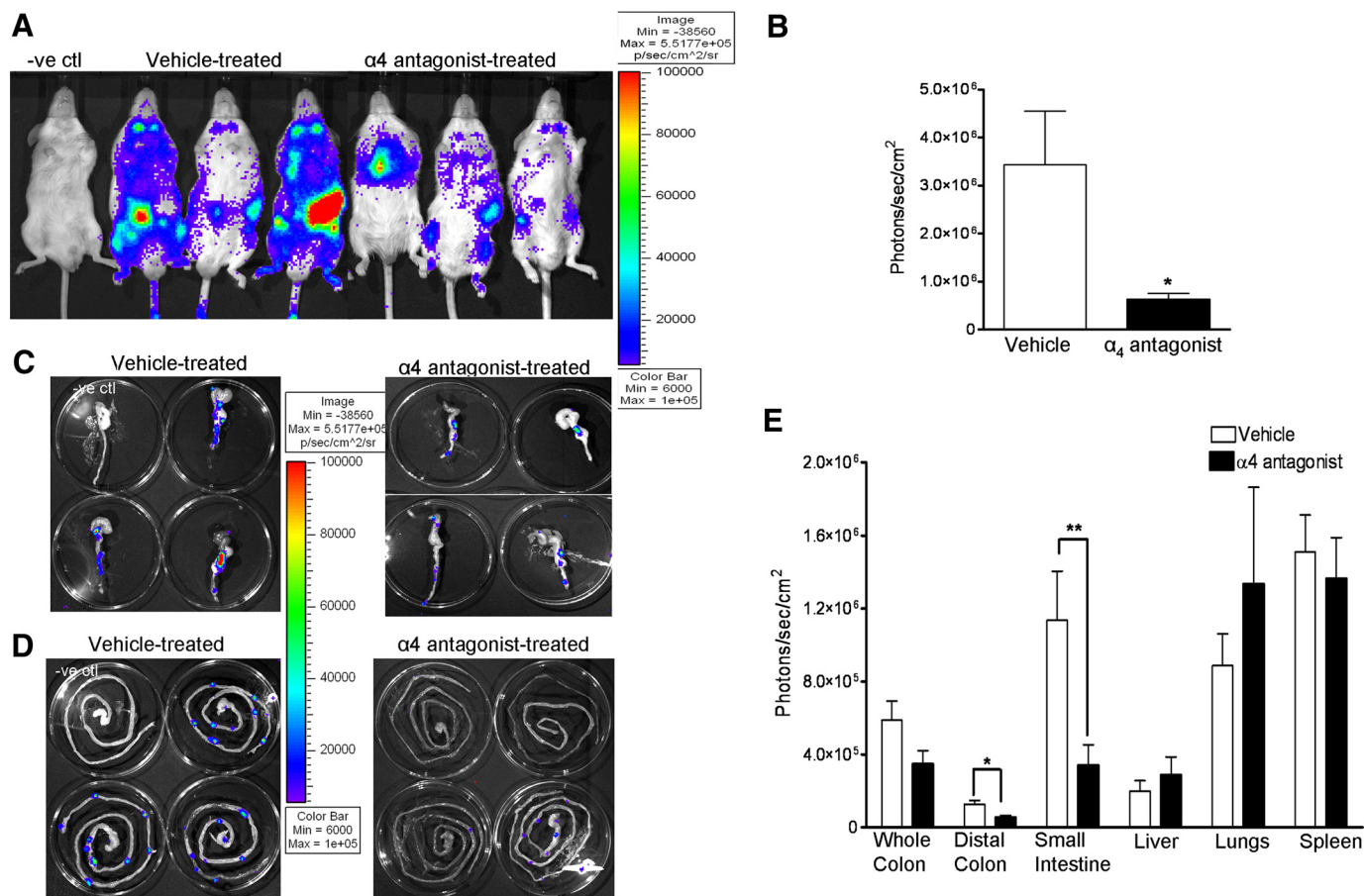


Figure 1. Effect of an α_4 -integrin antagonist on leukocyte trafficking in vivo using bioluminescence imaging. (A) Whole body bioluminescence imaging of FVB/N recipients with DSS-induced colitis treated with vehicle or α_4 -integrin antagonist 4 h post-adoptive transfer with *luc*⁺ cells. –ve ctrl, Non-recipient, non-DSS control. (B) Bioluminescence signal in the lower abdominal area of the α_4 -integrin antagonist- and vehicle-treated DSS mice. (C) Representative ex vivo images of colon and (D) small intestine (PPs) of DSS mice treated with vehicle and α_4 -antagonist. (E) Summary of bioluminescent signal in organs dissected from vehicle and α_4 -integrin antagonist-treated DSS mice. Data expressed as mean $\pm$ SEM; * $P \leq 0.05$, ** $P \leq 0.01$. DSS-treated groups, $n = 7/8$.

distal colons of the DSS recipients who were treated with the antagonist compared with the vehicle-treated recipients (Fig. 1C and E). Images of the small intestines revealed preferential homing of *luc*⁺ donor leukocytes to the PPs in the vehicle-treated mice, a phenomenon that was virtually absent in the compound-treated mice (Fig. 1D and E).

Treatment with the small molecule α_4 -integrin antagonist ameliorates inflammation in DSS-induced colitis

To assess therapeutic potential of the α_4 -integrin antagonist, an experimental model of colitis was used. DSS mice were orally gavaged with the compound (at 10, 30, or 90 mg/kg) or methyl cellulose (vehicle) twice daily from Day 3 up until Day 8. At Day 5 of the DSS treatment, the vehicle-treated mice began to experience progressive weight loss and increased DDAI score. In contrast, mice that received 10 mg/kg or 30 mg/kg compound lost significantly less weight from Day 5 than those that received the vehicle (Fig. 2A). In addition, all three concentrations of the α_4 -integrin antagonist rendered mice refrac-

tory to significant increases in DDAI compared with the vehicle group (Fig. 2B). At necropsy, a significant difference in colon length (Fig. 3A) and macroscopic score (Fig. 3B) was observed in the 30 mg/kg group compared with the vehicle-treated group. No significant differences in distal or proximal colonic weight were found among the three groups of compound-treated mice compared with vehicle-treated mice (data not shown). Administration of the three doses of the compound to non-DSS (control) mice showed no significant changes in health condition or macroscopic scores (Supplemental Figs. 1 and 2).

Histology of distal colons reveals amelioration of inflammation in mice treated with the small molecule α_4 -integrin antagonist

The colonic histological appearance in mice, exposed to 6 days of DSS and 3 days of water plus vehicle, was characterized by mucosal thickening, epithelial cell degeneration, edema, and infiltration of inflammatory cells into the mucosa and submucosa compared with non-DSS controls (Fig. 4A, C, and D),

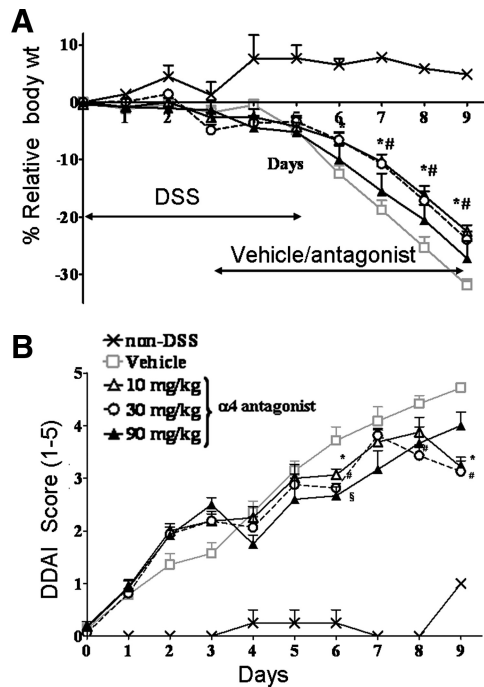


Figure 2. Therapeutic efficacy of the small molecule α_4 -integrin antagonist in DSS-induced colitis. (A) Percent relative body weight loss or gain from Day 0 in the different groups. Data expressed as mean $\pm$ SEM; * P < 0.05 vehicle versus 10 mg/kg; # P < 0.05 vehicle versus 30 mg/kg. (B) Stool consistency and fur texture/posture were used to generate a DDAI. Data expressed as mean $\pm$ SEM; * P < 0.05 vehicle versus 10 mg/kg; # P < 0.05 vehicle versus 30 mg/kg; § P < 0.05 vehicle versus 90 mg/kg. DSS-treated groups, n = 6–8; non-DSS controls, n = 2.

as reported previously [16]. The histology score and the extent of ulcerations were decreased significantly in the group administered 30 mg/kg of the α_4 -integrin antagonist when compared with vehicle-treated mice (Fig. 4A, B, D, and E). In the compound-treated group, we also observed a reduced infiltration of immune cells to the gut wall and increased epithelial regeneration (Fig. 4D and E). No significant improvement in histology score was observed in the low- or the high-dose, antagonist-treated group compared with vehicle-treated mice (Fig. 4A).

Gene expression analysis of α_4 -integrin antagonist-treated colonic tissue

To assess the effect of α_4 -integrin antagonist treatment on the expression of immune modulatory genes, total colonic RNA was isolated, and mRNA expression was evaluated using qRT-PCR. In the distal colon of DSS-treated animals that received vehicle, mRNA expression levels of the inflammatory mediators *IL-6*, *IL-8*, *COX-2*, *CCL-2*, *CCL-11*, and *VEGF* (data not shown) were up-regulated significantly compared with healthy controls, which is in agreement with previous studies [16, 25, 26]. As shown in Fig. 5, the DSS mice treated with 30 mg/kg compound showed significantly increased expression of *IL-17* and *CXCL10*, the regulatory T cell marker *FOXP3*, and the

transcription factor *T-bet* and *iNOS* compared with vehicle-treated mice. In addition, mice receiving 30 mg/kg α_4 -antagonist had significantly down-regulated colonic mRNA expression of *VEGF α* . A similar pattern was evident in the mice that received 10 mg/kg compound, with an additional increase in HSP70 (Fig. 5). No changes were seen in expression of the genes *IFN- γ* , *TNF- α* , *IL-1 β* , *IL-6*, *IL-8*, *IL-10*, *IL-11*, *IL-12*, *ROR γ* , *GATA-3*, *ITG α_4* , *PTGS2/COX-2*, *CCL-2*, *CCL-11*, and *CCL-20* in the compound-treated groups compared with the vehicle-treated group. In non-DSS (control) mice, the compound did not affect the mRNA expression of these genes, although a decrease in VEGF was observed in the 10 mg/kg group (Supplemental Fig. 3).

DISCUSSION

Current strategies for targeting leukocyte trafficking all involve large molecule biologics. Small molecule inhibitors, however, possess a number of attractive advantages over conventional antibody therapy. In contrast to antibodies, which need to be administered systemically, small molecules can be delivered orally, are cheaper to produce and less likely to elicit immunogenic responses [7, 8]. Efficacy of small molecule α_4 -integrin antagonism has been confirmed in a variety of autoimmune

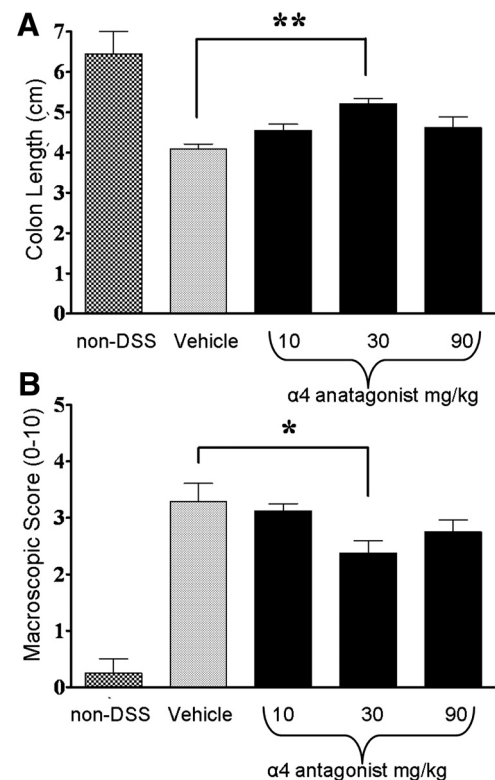


Figure 3. Macroscopic markers of inflammation reduced by the α_4 -integrin antagonist. On Day 9 of the study, the mice were killed, and colon length and weight were recorded. (A) Colon length expressed as cm. (B) Macroscopic score of colon. Data expressed as mean $\pm$ SEM; * P $\leq$ 0.05; ** P $\leq$ 0.01. DSS-treated groups, n = 6–8; non-DSS controls, n = 2.

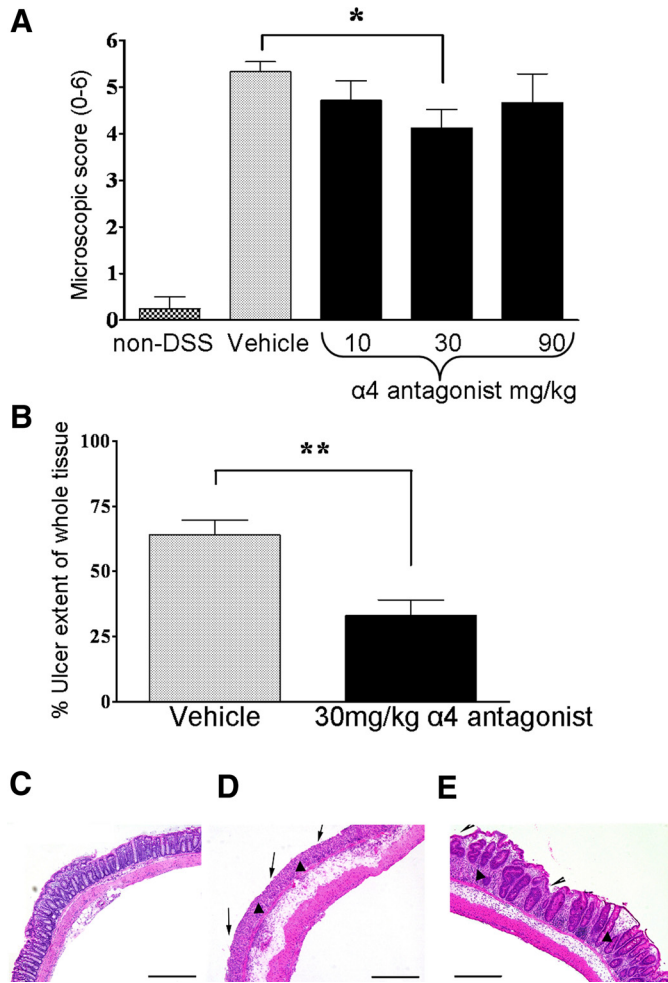


Figure 4. Treatment with 30 mg/kg of the α_4 -integrin antagonist induces histological recovery. (A) Histological scores, on a scale of 0–6, with 0 = no signs of damage, and 6 = severe inflammation and ulcerations of >75% of the tissue section in non-DSS control, vehicle, and α_4 -integrin antagonist-treated mice. (B) Extent of ulceration of total colonic tissue section in vehicle- and α_4 -integrin antagonist-treated mice. Data expressed as mean $\pm$ SEM; * $P \leq 0.05$; ** $P \leq 0.01$. DSS-treated groups, $n = 6$ –8; non-DSS controls, $n = 2$. Representative H&E tissue sections from (C) non-DSS control, (D) DSS mice that received vehicle (1% methylcellulose), and (E) DSS mice that received 30 mg/kg α_4 -integrin antagonist. Original magnification, 10 $\times$; original bars = 500 μ m. Arrows denote ulcerations, arrowhead regenerated epithelium, and filled triangle areas with high infiltration of immune cells and degenerated crypt structures in vehicle-treated mouse and decreased infiltration of cells and regenerated crypt structure in α_4 -integrin antagonist-treated mouse.

disease models [27, 28]. However, their potential as a treatment for IBD has yet to be investigated.

In this study, we demonstrate the therapeutic efficacy of a small molecule α_4 -integrin antagonist [14, 15] in an experimental model of colitis through blockade of leukocyte recruitment and induction of tissue healing. PK analysis of the α_4 -integrin antagonist demonstrated a consistent and rapid exposure of the compound in mice. Using in vivo bioluminescence imaging, we successfully monitored and quantified leukocyte

trafficking in a colitis model following adoptive transfer of *luc*⁺ cells from transgenic donors. Bioluminescence imaging is a relatively low cost, highly quantitative methodology that has been used to monitor infection progression, transgenic expression, tumor growth and metastasis, transplantation, toxicology, and gene therapy [29]. Several studies have shown robust correlation between cell numbers and bioluminescence signals [30, 31]. Using our novel bioluminescence trafficking model, we showed that treatment with a small molecule α_4 -integrin antagonist significantly reduced leukocyte recruitment to the inflamed distal colons and PPs compared with vehicle-treated animals, confirming the compound's ability to block leukocyte recruitment. To further evaluate the therapeutic efficacy of this compound, we also monitored body weight and a variety of clinical and macroscopic scores in DSS-induced colitic mice

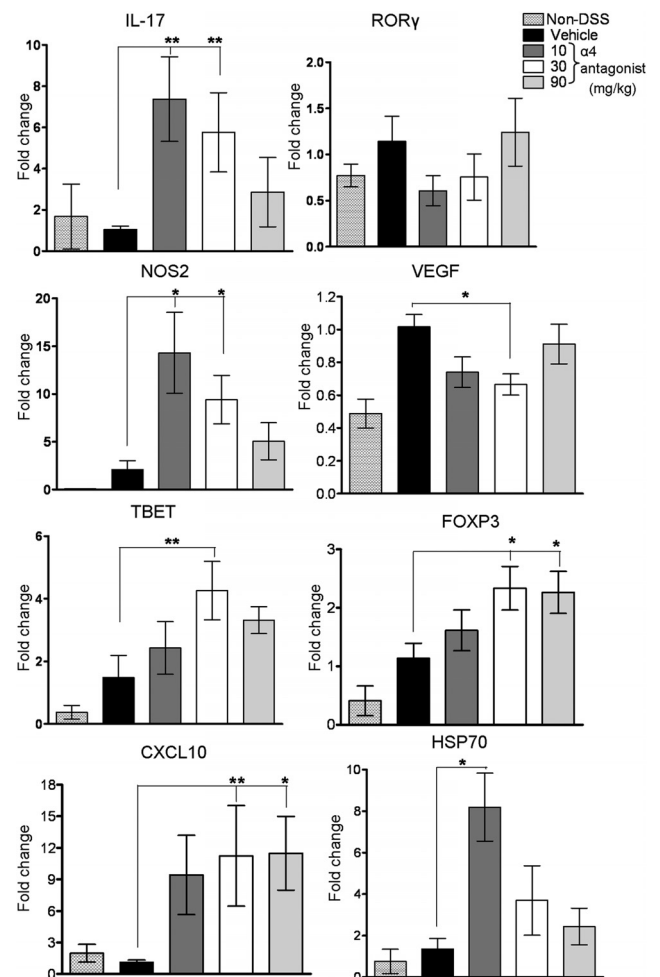


Figure 5. qRT-PCR analysis of gene expression in colonic tissue of vehicle-treated and α_4 -integrin antagonist-treated mice with DSS colitis. A range of genes involved in mediating inflammation and healing was examined by qRT-PCR, and the $2^{-\Delta\Delta C_t}$ method was used to calculate relative changes in gene expression compared with the vehicle-treated (control) group. *IL-6*, *IL-8*, *COX-2*, *CCL-2*, *CCL-11*, and *VEGF* are increased significantly in vehicle-treated DSS mice compared with non-DSS controls. Data expressed as mean $\pm$ SEM; * $P \leq 0.05$; ** $P \leq 0.01$. DSS-treated groups, $n = 6/7$; non-DSS controls, $n = 5$.

and observed significant improvement in animal health and macroscopic scores when compared with those DSS-treated mice administered with the vehicle.

As reported previously, acute inflammation in the DSS-treated animals that received vehicle was accompanied by significant up-regulation of *IL-6*, *IL-8*, *COX-2*, *CCL-2*, *CCL-11*, and *VEGF* compared with the non-DSS controls [16, 25, 26, 32]. Significant up-regulation of *IL-17* was evident in the 10 mg/kg and 30 mg/kg α_4 -antagonist-treated groups compared with the vehicle-treated group. Although *IL-17* is traditionally regarded as a proinflammatory cytokine, recent data suggest that it can also induce anti-inflammatory responses. For example, *IL-17* can inhibit *TNF- α* -induced RANTES gene expression [33] and mediates a protective function in intestinal inflammation [34, 35]. Interestingly, the transcription factor ROR γ t, a master regulator of Th17 cell differentiation and function [36], was not up-regulated simultaneously with *IL-17* in the compound-treated groups. This suggests that the cells producing *IL-17* in this setting are not a typical Th17 population. Supporting these observations, we detected a significant increase in *FOXP3* expression, coincident with the increase in *IL-17* levels, suggesting a change in T cell phenotypes within the tissue [37, 38].

Angiogenesis is recognized as having an integral role in IBD pathology, as suggested by the elevated levels of *VEGF* in CD and UC patients and in experimental colitis [39–42]. Down-regulation of *VEGF* in the colons of mice treated with 30 mg/kg compound suggests amelioration of tissue damage, which correlates with the histological findings. The decrease in *VEGF* expression was accompanied by a marked up-regulation of *iNOS* in the colons of these mice. Notably, a role for *iNOS* in wound healing [43, 44], in homeostatic regulation of leukocyte recruitment [45], and most recently, in the healing of DSS colonic lesions [46] has been demonstrated. The gene expression and histological data suggest that attenuation of DSS colitis by the α_4 -integrin antagonist is associated with restoration of colonic tissue integrity, possibly through the down-regulation of angiogenesis and increased expression of genes associated with tissue healing. Future studies should address the contribution of specific immune cell populations, which express $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins, to disease pathogenesis and its amelioration by the α_4 -integrin antagonist.

In conclusion, we have demonstrated that mice administered with a small molecule α_4 -integrin antagonist are protected against colitis via blockade of leukocyte recruitment and through possible promotion of tissue healing. The data presented here clearly demonstrate that small molecule α_4 -integrin antagonists are a promising alternative strategy for treating IBD. Additionally, we show that bioluminescence imaging is a powerful technique for preclinical evaluation of potential therapeutic molecules for other autoimmune diseases.

AUTHORSHIP

C.T.M.: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, and statistical analysis. G.M.: acquisition of data, analysis and interpretation of data, and technical or material support. J.M.: ac-

quisition of data, analysis and interpretation of data, statistical analysis, and technical or material support. A.H.: technical or material support. E.F.: technical or material support. A.Q.: technical or material support. P.G.M.: study concept and design and analysis and interpretation of data. K.L.: obtained funding. L.O.: obtained funding. F.S.: critical revision of the manuscript for important intellectual content and obtained funding. S.M.: study concept and design, acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, and study supervision. K.N.: study concept and design, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, obtained funding, and study supervision.

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