Oral treatment with a novel small molecule alpha 4 integrin antagonist, AJM300, prevents the development of experimental colitis in mice

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Received 5 December 2012; received in revised form 11 March 2013; accepted 28 March 2013

Abstract

Background and aims: Inhibition of lymphocyte trafficking by treatment with an anti-α4 integrin antibody has been clinically validated as a therapeutic approach for inflammatory bowel disease (IBD), and the orally effective 'anti-α4 integrin therapy' may be more convenient in clinical practice. The aim of this study was to investigate the pharmacological profile and anti-inflammatory effect of a novel, orally active small molecule α4 integrin antagonist, AJM300.

Methods: The binding specificity/potency of HCA2969 (the active metabolite of AJM300) were investigated in vitro. The pharmacodynamics for α4 integrin antagonism of AJM300 was investigated in mice. The anti-inflammatory effect of AJM300 fed in a diet and the anti-α4 integrin monoclonal antibody was evaluated in a mouse colitis model induced by transfer of IL-10 deficient T cells.

Results: HCA2969 selectively inhibited the in vitro binding of α4 integrin (α4β7/α4β1) to the cell adhesion molecules. Oral treatment with AJM300 dose-dependently inhibited lymphocyte homing to Peyer's patches and increased the peripheral lymphocyte count in the same dose range. AJM300 dose-dependently prevented the development of experimental colitis in mice. A significant inhibition of colon weight increase was accompanied by inhibition of T-cell

KEYWORDS
α4 Integrin antagonist; Oral drug; AJM300; Inflammatory bowel disease; Lymphocyte homing

Abbreviations: IBD, inflammatory bowel disease; AJM300; orally active small molecule α4 integrin antagonist; AJM300M1, a special formulation containing AJM300 at approximately 43% and excipients; AJM300M1 preparation; VCAM-1, vascular cell adhesion molecule-1; MAdCAM-1, mucosal addressin cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; PK, pharmacokinetics; PD, pharmacodynamics; HEK, human embryonic kidney; PBS, phosphate-buffered saline; SCID, severe combined immuno-deficient; CTRL, control; IL-10, interleukin-10; DAPI, 4′,6-diamidino-2-phenylindole.

☆ Conference presentation: GASTRO2009-UEGW/WCOG London Poster: # P0270, 'Best poster'.
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http://dx.doi.org/10.1016/j.crohns.2013.03.014
1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract. Although the cause of the disease remains unknown, the infiltration of lymphocytes into the lamina propria at the sites of gastrointestinal inflammation is a well-documented pathogenic mechanism of IBD. It has been suggested that infiltration of lymphocytes is accelerated by increased expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on the surface of endothelial cells at the site of inflammatory lesions and that the release of cytokines and inflammatory mediators from the infiltrating inflammatory cells leads to further exacerbation of inflammation. The binding of lymphocytes to VCAM-1 or MAdCAM-1 requires the expression of specific integrins, α4β1 or α4β7 integrin, respectively. VCAM-1 is expressed on the luminal surface of endothelium in numerous types of tissues while MAdCAM-1 is expressed predominantly in the intestinal lamina propria.

Several studies in various animal models of colitis have suggested that α4β1 and α4β7 integrins and their ligands VCAM-1 and MAdCAM-1 are attractive molecular targets for the treatment of IBD. Treatment with an antibody specific for α4 or α4β7 integrin suppressed the development of spontaneous colitis in the cotton-top tamarin and MAdCAM-1/Fc chimera protein (MAdCAM-1/Fc monomorphic antibody). In addition, α4 integrin blockade has been validated as a therapeutic approach for IBD in clinical studies with anti-α4 integrin antibody, natalizumab, or anti-α4β7 integrin antibody, vedolizumab. Furthermore, anti-β7 integrin, etrolizumab, and anti-MAdCAM-1 antibodies are being developed for the treatment of ulcerative colitis. However, there will be some concerns regarding the long-term administration of these agents, since adverse effects specifically related to biologics, such as immunogenicity, infusion site reaction, and the loss of efficacy related to the production of persistent antibodies against biologics, have been observed in the treatment with anti-TNF antibodies. Therefore, the development of orally active α4 integrin antagonists is a reasonable and preferable approach. Actually, several small molecule α4 integrin antagonists is a reasonable and preferable approach. Several studies in various animal models of colitis have suggested that α4β1 and α4β7 integrins and their ligands VCAM-1 and MAdCAM-1 are attractive molecular targets for the treatment of IBD. α4 integrin antagonism related to lymphocyte homing to Peyer's patches and peripheral lymphocyte counts were investigated in mice. In addition, the efficacy of AJM300 was evaluated in a mouse model of colitis induced by the adoptive transfer of IL-10 deficient CD4+ T cells and the efficacy was compared to that of treatment with an anti-α4 integrin monoclonal antibody.

2. Materials and methods

2.1. Compounds

AJM300, HCA2969 (an active metabolite of AJM300) and 3H-HCA2969 were used for in vitro studies. AJM300M1 preparation (AJM300M1: a special formulation containing AJM300 at approximately 43% and excipients) was used for oral studies. These compounds were synthesized and provided by the Research Center of Ajinomoto Pharmaceutical Co., Ltd. (Kawasaki, Japan). AJM300 and HCA2969 (an active metabolite of AJM300) were a gift from Dr. T. Tanaka.

2.2. Proteins, antibodies and cell lines

HEK293 cells producing recombinant rat MAdCAM-1/Fc chimeric protein (rMAdCAM-1/Fc) were a gift from Dr. T. Tanaka. The rMAdCAM-1/Fc was purified from the supernatant of the HEK293 cell line using a protein G column. Recombinant human VCAM-1/Fc chimera protein (hVCAM-1/Fc), mouse VCAM-1/Fc chimera protein (mVCAM-1/Fc) and human ICAM-1/Fc chimera protein (hICAM-1/Fc) were purchased from R&D Systems (Minneapolis, MN). Recombinant mouse MAdCAM-1/Fc chimera protein (mMAdCAM-1/Fc) was purchased from Genzyme Techné (Cambridge, MA). The α4β1 integrin-expressing human T-cell line (Jurkat), α4β7 integrin-expressing basal oropharyngeal leukemia cell line (RBL-2H3), α4β7 integrin-expressing mouse lymphoma cell line (TK-1), α4β7 integrin-expressing mouse lymphoma cell line (RBL-1) were purchased from American Type Culture Collection (Manassas, VA). The α4β1 integrin-expressing mouse lymphoma cell line (L1-2) and the α4β7 integrin-expressing human B-cell lymphoma cell line (RPMI-8866) were kind gifts from Drs. H. Kikutani and M. Miyasaka (Osaka University, Osaka, Japan). These cells were cultured in RPMI1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (MP Biomedicals, Santa Ana, CA) in a humidified 5% CO2 incubator at 37 °C. Anti-mouse α4 integrin monoclonal antibody was purified by affinity chromatography using protein G column from ascites fluid from a mouse injected with the hybridoma cell line producing anti-mouse α4 integrin (PS/2).
2.3. Affinity of $^3$H-HCA2969 for $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrin

Jurkat, RPMI-8866 or TK-1 cells (1.8 x 10^6 cells each) were incubated with serially diluted $^3$H-HCA2969 solution (final concentration 0.03 to 4 nmol/L) in the presence of 2 mmol/L Mn^2+ at 25 °C for 40 min. After incubation, the preparation was centrifuged (4000 rpm, 3 min) and the cells were resuspended in wash buffer (Tris-Buffered Saline containing 0.1% bovine serum albumin 2 mmol/L D(+)-glucose and 2 mmol/L Mn^2+). Cells were washed again with wash buffer, and Lumasafe Plus (LUMAC-LSC B.V., Groningen, Nether-lands) was added to the vials and mixed. The radioactivity of each sample (counts per minute, cpm) was determined for 2 min with a liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA) to estimate the amount of $^3$H-HCA2969 bound to the cells. The specific binding was determined by subtraction of the non-specific binding which represented the residual radioactivity after the addition of an excess amount of non-labeled HCA2969 to each reaction mixture. The specific dissociation constant (K_d value) was calculated using GraphPad Prism software (GraphPad Software Inc., San Diego, CA) and is expressed as the mean ± SEM of 3 independent experiments.

2.4. Cell adhesion

The chimeric adhesion molecule proteins (hVCAM-1/Fc, mVCAM-1/Fc, mMAdCAM-1/Fc, mMAdCAM-1/Fc or hICAM-1/ Fc) were immobilized onto flat-bottomed 96-well plates and incubated at 4 °C overnight. On the following day, each well was washed with phosphate buffered saline (PBS) and non-specific binding was blocked using blocking buffer (PBS buffer containing Block Ace powder®, DS Pharma Biomedical Co., Ltd. Osaka, Japan) followed by incubation at room temperature for at least 2 h. Either $\alpha 4\beta 1$, $\alpha 4\beta 7$ or $\alpha L\beta 2$ integrin-expressing cells ($5 \times 10^4$/well) were treated with HCA2969 in the assay buffer (Dulbecco’s modified Eagle medium containing 20 mmol/L HEPES, 0.1% bovine serum albumin and 0.4 mmol/L MnCl_2) and added to wells pretreated with the chimeric adhesion molecule protein, followed by incubation at room temperature for 40 to 60 min. Then wells were washed and the bound cells were solubilized with Triton X-100 detergent (Sigma-Aldrich Japan K.K., Tokyo, Japan) followed by incubation at room temperature for at least 2 h. Either $\alpha 4\beta 1$, $\alpha 4\beta 7$ or $\alpha L\beta 2$ integrin-expressing cells ($5 \times 10^4$/well) were treated with HCA2969 in the assay buffer (Dulbecco’s modified Eagle medium containing 20 mmol/L HEPES, 0.1% bovine serum albumin and 0.4 mmol/L MnCl_2) and added to wells pretreated with the chimeric adhesion molecule protein, followed by incubation at room temperature for 40 to 60 min. Then wells were washed and the bound cells were solubilized with Triton X-100 detergent (Sigma-Aldrich Japan K.K., Tokyo, Japan). To determine the number of bound cells, each well was used for CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega K.K., Tokyo, Japan) by colorimetry of the activity of lactate dehydrogenase. The 50% inhibitory concentration (IC50) was measured.

2.5. Lymphocyte homing

After mesenteric lymph nodes were collected from BALB/c mice (9 weeks of age, female), single cells were prepared and stained with a fluorescent dye PKH26 (Zynaxis, Inc., Malvern, PA). AJM300M1 solution was prepared with distilled water just before use, mixed with a stirrer to keep the solution homogenous and used within 1 hour after preparation. Thirty minutes after a single oral administration of AJM300M1 at doses of 0.3, 1.1, 3, 10 or 30 mg/kg (expressed as a dose of AJM300 or placebo to BALB/c mice (8 weeks of age, female), the fluorescent-labeled mesenteric lymph node cells ($4 \times 10^6$ cells) were injected into the caudal vein. One hour after injection of the cells, mice were euthanized under ether anesthesia, and Peyer’s patches were collected and a single cell suspension was prepared. These single cell suspensions ($3 \times 10^6$ cells) were analyzed using a Fluorescent Activated Cell Sorter (Nippon Becton Dickinson Company Ltd., Tokyo, Japan). The percentage of fluorescent-labeled cells in the Peyer’s patch cells was measured.

2.6. Peripheral lymphocyte counts

To evaluate the efficacy of AJM300 on the peripheral lymphocyte count in normal mice, a single oral administration of AJM300M1 was performed at dose of 0.3, 3, 30, or 300 mg/kg (expressed as a dose of AJM300) or placebo, to BALB/c mice (7 weeks of age, male). AJM300M1 solution was prepared with distilled water just before use, mixed with a stirrer to keep the solution homogenous and used within 1 hour after preparation. Ninety minutes after administration, cardiac blood samples were collected under ether anesthesia.

The number of lymphocytes in the peripheral blood was counted using an automated hematology analyzer (Sysmex corporation, Hyogo, Japan: SF-3000).

2.7. Experimental colitis model

The anti-inflammatory efficacy of AJM300 was evaluated in a well-validated mouse model of colitis induced by adoptive transfer of IL-10 deficient CD4^+ T cells. CD4^+ T cells were isolated from spleens and mesenteric lymph nodes of diseased IL-10^−/− BALB/c mice (21 to 35 weeks of age, male) and adoptively transferred to female C.B-17/lcr-scid/scid Jcl (SCID) mice (8 weeks of age, female). To evaluate the efficacy of AJM300, AJM300M1 or placebo was fed ad libitum in the diet, containing a concentration of AJM300 at 0.03, 0.1, 0.3 or 1% (expressed as a concentration of AJM300M1) from day −1. To compare the efficacy of AJM300 to the $\alpha 4$ integrin antibody, the anti-mouse $\alpha 4$ integrin monoclonal antibody (PS/2) was administered intraperitoneally at a dose of 10 mg/kg twice a week starting on day −1. The stool consistency (0: normal beaded stool; 1: soft stool; 2: diarrhea) was recorded every day, starting from day 5. On day 15, after euthanasia under ether anesthesia, the colons were removed and weighed. The infiltration of CD3^+ T cells in the lamina propria was analyzed by immunofluorescence staining and histological scoring was performed as described below. To evaluate the relationship between PK and efficacy of AJM300, a concentration of 0.1%, 0.3% or 1% AJM300M1 were fed ad libitum in the diet from day −1 and cardiac blood was collected at 10 AM, 2 PM, 8 PM and midnight on day 14 and at 6 AM and 10 AM on day 15.
2.8. Histopathologic scoring and immunofluorescence staining

Colons were removed from colitis model mice on day 15 and placed in formalin or sucrose. Formalin-fixed tissues were embedded in paraffin. Paraffin-embedded specimens (2–3 μm thick) were stained with hematoxylin and eosin (H&E), or periodic acid-Schiff (PAS). Blinded histopathological analysis was performed using the following scoring system (modified from Davidson et al., 1996)36: i) degree of mononuclear cell infiltration in lamina propria (scored between 0 and 3); ii) goblet cell loss (scored between 0 and 3); iii) epithelial hyperplasia (scored between 0 and 3). The total histopathological score (between 0 and 9) was calculated by combining each score. Sucrose-permeated tissues were embedded in OCT and cooled in liquid nitrogen, and stored at –80 °C. Cryostat specimens (6 μm thick) were air-dried, fixed in 2% paraformaldehyde, and incubated with 0.3% hydrogen peroxide. After pretreatment with blocking agent consisting of Block Ace® (DS Pharma Biomedical Co., Ltd., Osaka, Japan), sections were incubated for 60 minutes with the primary antibody, hamster anti-mouse CD3e (BD Pharmingen™, San Diego, CA). After washing in PBS, the sections were incubated for 30 min with peroxidase-conjugated goat anti-hamster IgG (H + L) antibody, followed by TSA™ cyanine 3 systems (PerkinElmer, Inc., Waltham, MA), and then mounted using Vectashield® Mounting Medium with DAPI (Vector Laboratories, Inc. Burlingame, CA). The rate of T cell infiltration (stained with cyanine 3) per area was calculated as a percentage of the lamina propria selected within the blue fluorescence image (stained with DAPI) using image editing software, Win Roof ver.5.3 (Mitani Corporation, Tokyo, Japan).

2.9. Concentration of HCA2969 in plasma

Plasma samples from cardiac blood were centrifuged (10,000 rpm, 10 min, 4 °C) and processed by solid-phase extraction. The concentration of HCA2969 in plasma was measured using liquid chromatography–tandem mass spectrometry (LC–MS/MS).

2.10. Animals

IL-10−/− BALB/c mice and wild type BALB/c mice were obtained from Charles River Laboratories Japan, Inc. (Tokyo, Japan). C.B-17/lcr-scid/scid Jcl (SCID) mice were obtained from Charles River Laboratories Japan, Inc. (Tokyo, Japan). All mice were housed and bred under specific pathogen free conditions. All procedures were performed in accordance with the institutional Animal Care and Use Committee of the Research Center of Ajinomoto Pharmaceuticals Co., Ltd.

2.11. Statistical analyses

IC50 values from in vitro assays, EC50 and EC90 values from homing assays and the other statistical analysis described below were calculated using the statistic analysis software, SAS System ver. 8.2 (SAS Institute Japan Ltd., Tokyo, Japan). In homing assays, the concentration of HCA2969 in the placebo-treated group (control group) was converted to ‘0.1 ng/mL’ before the logarithmic transformation for statistical analysis using a sigmoidal Emax model. On peripheral lymphocyte counts, Steel test was used for statistical analysis. In the mouse colitis model, Welch’s t-test or Student’s t-test was used for statistical analysis of the comparison between placebo treated group (control group) and the non-T cell transferred group (non-transferred group). Parametric Dunnett’s multiple comparison test was used for statistical analysis of the efficacy of AJM300 on the colon weight and the area ratio of CD3+ T cells in lamina propria. A parametric Tukey multiple comparison test was used for statistical analysis of the comparison of the efficacy between AJM300 and the anti-mouse α4 integrin monoclonal antibody (PS/2) on colon weight and histopathological scores, a non-parametric Steel–Dwass’ test was used on the area ratio of CD3+ T cells in lamina propria. A value of p < 0.05 was considered statistically significant.

### Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Integrins</th>
<th>Cell lines</th>
<th>K0 (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>α4/11</td>
<td>Jurkat</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>Human</td>
<td>α4/17</td>
<td>RPMI-8866</td>
<td>0.46 ± 0.07</td>
</tr>
<tr>
<td>Mouse</td>
<td>α4/17</td>
<td>TK-1</td>
<td>0.20 ± 0.03</td>
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</table>

The dissociation constant (K0) values were determined in the assay for binding of 3H-HCA2969 to human α4/11 integrin, human α4/17 integrin and mouse α4/17 integrin using cell lines. The K0 values were expressed as the mean ± SEM of 3 independent experiments.

3. Results

3.1. Characterization of the biochemical properties of HCA2969 (the active metabolite of AJM300)

To evaluate the affinity of HCA2969 for α4 integrin, the binding of 3H-HCA2969 to cells expressing α4/1 integrin (Jurkat) or α4/7 integrin (RPMI-8866 or TK-1) was assessed (Table 1). 3H-HCA2969 bound to human α4/1 integrin and human α4/7 integrin with K0 values of 0.32 ± 0.01 nmol/L and 0.46 ± 0.07 nmol/L, respectively. The affinity was similar to that of mouse α4/7 integrin (K0 value; 0.20 ± 0.03 nmol/L). These data indicated that HCA2969 was a chemical compound with a high affinity for α4 integrin and that there was no inter-species difference in the affinity to α4/7 integrin between humans and mice.

Second, the inhibitory activity of HCA2969 to the binding of α4/1 or α4/7 integrin to the respective ligand was evaluated using an in vitro cell binding assay (Table 2). HCA2969 inhibited the binding of human α4/1 integrin-expressing cells (Jurkat) to human VCAM-1 in a concentration-dependent manner with an IC50 value of 5.8 ± 1.6 nmol/L. HCA2969 also inhibited binding of human α4/7 integrin-expressing cells (RPMI-8866) to rat MadCAM-1 (IC50 value; 1.4 ± 0.3 nmol/L). In contrast, the IC50 value for the binding of human αL/β2 integrin-expressing cells (Jurkat) to human ICAM-1 was 54,000 ± 11,000 nmol/L. Furthermore, HCA2969 did not show the affinity to various receptors, channels and transporters (Supplemental Table 1A), and any inhibition of the enzyme activity and the secretion of human enzymes was confirmed.
of cytokines and inflammatory mediators (Supplemental Table 1B), indicating that HCA2969 was a highly selective dual α4β1/α4β7 integrin antagonist.

Third, to evaluate the inter-species difference in the inhibitory activity of HCA2969 for cell binding in vitro, the cell binding assay was performed using mouse or rat-derived cell lines. HCA2969 inhibited the binding of mouse or rat α4β1 integrin-expressing cells to VCAM-1 (IC50 values; 0.94 ± 0.33 nmol/L and 8.5 ± 1.8 nmol/L, respectively). HCA2969 also inhibited the binding of mouse or rat α4β7 integrin-expressing cells to MadCAM-1 (IC50 values; 26 ± 1 nmol/L and 4.1 ± 1.2 nmol/L, respectively) (Table 2). There was no inter-species difference in the inhibitory activity of HCA2969 on the binding of α4 integrin to their ligand in vitro among human, mice and rats.

### Table 2  Inhibitory effects and selectivity of HCA2969 on binding between α4 integrin-expressing cells and soluble cell adhesion molecules.

<table>
<thead>
<tr>
<th>Species</th>
<th>Integrins</th>
<th>Cell lines</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>α4β1</td>
<td>Jurkat</td>
<td>VCAM-1</td>
</tr>
<tr>
<td></td>
<td>α4β7</td>
<td>RPMI-8866</td>
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<td>Mouse</td>
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</tr>
<tr>
<td></td>
<td>α4β7</td>
<td>RBL-1</td>
<td>MadCAM-1</td>
</tr>
</tbody>
</table>

The activity of HCA2969 to inhibit the binding of integrins to cell adhesion molecules was evaluated in the cell binding assay using cell lines. The 50% inhibitory concentration (IC50) was expressed as the mean ± SEM of 3 or 4 independent experiments.

3.2. Inhibition of lymphocyte homing and increased peripheral lymphocyte counts after oral administration of AJM300

Lymphocyte homing to Peyer’s patches was inhibited in a dose-dependent manner after a single oral administration of AJM300M1 in mice. A correlation was observed between the concentration of HCA2969 in plasma and the ratio of homing lymphocytes to Peyer’s patches. Using a sigmoidal Emax model, the 50% and 90% effective concentrations (EC50 and EC90) were determined as 100 ng/mL (180 nmol/L) and 680 ng/mL (1200 nmol/L), respectively (Fig. 1).

The effect of AJM300 on the peripheral lymphocyte counts was also determined in mice. Peripheral lymphocyte counts were dose-dependently increased after a single oral administration of AJM300M1 (Fig. 2).

3.3. Prevention of the development of murine experimental colitis induced by adoptive transfer of IL-10 deficient CD4+ T cells after oral administration of AJM300 in the diet

AJM300 prevented an increase in both the stool consistency score (Fig. 3A) and colon weight (Fig. 3B) in a mouse model of colitis induced by adoptive transfer of IL-10 deficient CD4+ T cells, dose-dependently. Inhibition of an increase in colon weight was statistically significant in experimental groups fed a diet containing 0.1, 0.3 and 1% AJM300M1 (p < 0.001). A significant inhibition (>95%) of T-cell infiltration to the lamina propria was observed in mice fed a diet containing 1% AJM300M1 compared to the control group (Fig. 3C). Pro-inflammatory cytokines (IFN-γ, IL-17 and TNF-α) in the colon samples were clearly reduced in mice fed a diet containing 1% AJM300M1 compared to the control group (Supplemental Table 2). In addition, the efficacy of the 1% AJM300M1 was comparable with anti-α4 integrin

![Figure 1](image1)

**Figure 1** Relationship between total plasma concentration and lymphocyte homing into the Peyer’s patches after oral administration of AJM300M1 at doses of 0.3, 1.1, 3, 10 or 30 mg/kg in normal mice. Each point (○) represents the relationship between the ratio of fluorescence-labeled lymphocytes homing into the Peyer’s patches and the concentration of HCA2969 in plasma. The line presents the fit to a simple Emax model. CTRL, placebo-treated (○).

![Figure 2](image2)

**Figure 2** AJM300 increased peripheral lymphocyte counts in normal mice. Lymphocyte numbers in cardiac blood of BALB/c mice were measured at 90 min after oral administration of AJM300M1 at doses of 0.3, 3, 30 or 300 mg/kg (▲) or placebo (○). Data are represented as mean value ± SEM. (n = 8). *p < 0.05, **p < 0.01 compared with the placebo-treated group (CTRL) by Dunnett’s test. Each point (○) represents the ratio of fluorescence-labeled lymphocytes homing into the Peyer’s patches after oral administration of AJM300M1 at doses of 0.3, 1.1, 3, 10 or 30 mg/kg in normal mice. CTRL, placebo-treated (○).
monoclonal antibody (PS/2) treatment at a dose of 10 mg/kg administered twice weekly (Fig. 4A–C), where the saturation of α4 integrin blockade was maintained for at least 4 days per dose (data not shown). This effect was accompanied by a
A novel α4 integrin antagonist inhibits experimental colitis

Figure 5 1% AJM300M1 in the diet of mice prevented the development of colitis upon histological examination. (A) Histopathological scoring of severity of colitis was performed in colons from mice. Data represent the mean ± SEM. (n = 8). **p < 0.001 compared to control mice not receiving cell transfer (non-transferred) by Welch’s test. NS, not significantly different from the anti-α4 integrin monoclonal antibody (PS/2) treated group, ***p < 0.01 compared to the placebo-treated group (CTRL) by Tukey–Kramer’s test. (B) Hematoxylin and eosin (i, ii, iii, iv) and PAS (v, vi, vii, viii) staining of colon. Representative images of the colon from placebo treated mouse (i, v), an AJM300M1-treated mouse (ii, vi), an antibody to α4 integrin-treated mouse (iii, vii) and a control SCID mouse which did not receive cell transfer (iv, viii).

4. Discussion

This is the first report to our knowledge describing an orally active small molecule α4 integrin antagonist, with anti-inflammatory effects on an experimental colitis. The maximum efficacy of AJM300 was comparable to that of the saturated α4 integrin blockade achieved by antibody treatment.

This study demonstrated that HCA2969, the active metabolite of AJM300, was a specific, dual α4β1/α4β7 integrin antagonist. HCA2969 had the similar affinities for α4β1 and α4β7 integrins and inhibited the binding of α4β1/α4β7 integrin-expressing cells to VCAM-1/MAdCAM-1 in vitro. Since the K0 value for human α4β1 integrin was similar to that for human α4β7 integrin, the difference between mouse α4β1 and α4β7 integrins in IC50 value for the cell-binding might be due to the difference in the assay system (e.g. expression level of the integrin on the cell). Potentially, because of high selectivity of AJM300 to α4 integrin, AJM300 might not have the risk of the side effect associated with the inhibition of αLβ2 integrin and the inhibition of the other various signals and activities related to immune system, for example, the increase in the risk of infection caused by the inhibition of neutrophil migration.37 Whereas, the blockade of α4 integrin (α4β1/α4β7) leads to the inhibition of the lymphocyte migration mediated by the binding to MAdCAM-1 as well as VCAM-1. Indeed, an α4 integrin blockade with antibody (natalizumab) has successfully

Figure 6 The plasma concentration of HCA2969 from AJM300M1-treated mice with colitis. AJM300M1 at concentrations of 0.1% (▲), 0.3% (▼) or 1% (♦) was fed to mice ad libitum with the diet from day −1. Cardiac blood was collected at 10 AM, 2 PM, 8 PM and midnight on day 14 and at 6 AM and 10 AM on day 15. The plasma concentration of HCA2969 was measured. Each dashed line indicates the EC50 (= 100 ng/mL) or the EC90 (= 680 ng/mL) value for lymphocyte homing. Data represent the mean value ± SEM. (n = 4 for each group).
observed. After the launch of the gut-specific vedolizumab production of persistent antibodies against biologics, infusion site reaction, and the loss of efficacy related to the specifically related to biologics, such as immunogenicity, long-term administration of these agents. Adverse effects against biologics. While some biologics have been in clinical under careful consideration as much as PML of these gut-selective anti-integrin antibodies should be alleviated inflammation in the gastrointestinal tract with its mechanism of action; interference with the function of both α4β7 (natalizumab) could inhibit the homing of leucocytes to the intestine as well as the central nervous system (CNS). The mechanistic basis for the recrudescence of JC virus infection culminating in PML is presumably due to impaired immune surveillance of the CNS. It has been reported that positive status with respect to anti-JC virus antibodies, prior use of immunosuppressants and prolonged treatment of natalizumab, alone or in combination were associated with distinct levels of PML risk. In fact, based on the huge post-marketing surveillance data derived from approximately 100,000 natalizumab-treated patients, natalizumab-associated PML has not been observed with therapy of 6 months or less and no case of PML has been reported in a patient who was negative for anti-JC virus antibodies. AJM300 has the potential risk for PML, however, to date, the case has not been observed in the clinical trials. The potential PML risk may be minimized in the induction therapy within 6 months or in the maintenance considering the risk factor for natalizumab-associated PML (e.g. measuring anti-JC virus antibodies). In addition, AJM300 has a much shorter pharmacological half-life (data not shown) than natalizumab (approximately 11 days) and the potential advantage is that the early removal of drug from the body after onset of PML could lead to more favorable outcomes of the complication, but this is a hypothesis that remains to be proved. On the other hand, gut-selective anti-inflammatory effect without systemically inhibiting adaptive immune responses theoretically believes to provide immunity to PML. Targeting the α4β7 integrin (vedolizumab) exclusively will alleviate inflammation in the gastrointestinal tract with minimizing undesired effects in other organs. Blockade of α4β7 and αEβ7 (etrolizumab) could regulate trafficking in the intestine as well as retention exclusively on mucosal intraepithelial of immune cells. This ‘theoretical’ risk for PML of these gut-selective anti-integrin antibodies should be proved in clinical under careful consideration as much as anti-α4β7/α4β1 integrin antibody. Second, oral drug formulations often provide good drug adherence and avoid the loss of efficacy related to the production of persistent antibodies against biologics. While some biologics have been in clinical trial, there could remain some concerns regarding the long-term administration of these agents. Adverse effects specifically related to biologics, such as immunogenicity, infusion site reaction, and the loss of efficacy related to the production of persistent antibodies against biologics, could be observed. After the launch of the gut-specific vedolizumab or etrolizumab, the patients with loss of response or intolerance may be observed in the treatment with the anti-integrin antibodies. Oral treatment with small molecules could be beneficial to the patients with IBD by circumventing these drawbacks. For the patients who respond to the anti-integrin antibody, the oral treatment with small molecule which has the same mechanism of action as the prior efficacious treatment with biologics and the availability only if the safety is established, may be favorable option.

Next, oral treatment with AJM300 dose-dependently inhibited mesenteric lymphocyte homing into Peyer’s patches in mice. When the concentration of HCA2969 in plasma was maintained over the EC90 value, the inhibition of lymphocyte homing was comparable to that of pre-treatment of the cells with anti-α4 integrin antibody in mouse (data not shown). The phenomenon of lymphocyte homing regulates the generation and propagation of immune responses and ensures the coordinated expression of immunological functions by the lymphoid tissues in vivo. Previous study reports suggested that α4β7 integrin may appear to be essential for lymphocyte homing into Peyer’s patches as well as for lymphocyte infiltration into the site of inflammatory lesions.

Oral treatment with AJM300 prevented the development of colitis induced by transfer of IL-10 deficient CD4+ T cells in mice. In this model, the anti-inflammatory effect due to the inhibition of CD4+ T cell infiltration into the colon potentially might result in the prevention of development of colitis and diarrhea. It has been suggested that the maintenance of saturated occupancy of α4 integrin was required for maximum efficacy of α4 integrin antagonist in autoimmune disease models as well as in a natalizumab clinical study for Crohn’s disease. In our preliminary study, the maintenance of saturated occupancy of α4 integrin was achieved with anti-α4 integrin monoclonal antibody (PS/2) treatment at a dose of 10 mg/kg twice weekly in normal mice (data not shown). Unfortunately, it was technically difficult for AJM300 to measure the occupancy of HCA2969 because of its rapid dissociation rate. Instead of the saturated occupancy, the maintenance of concentration of HCA2969 in plasma over the EC90 value for lymphocyte homing was used for determination of dosing regimen. AJM300 administrated with a 1% AJM300M1 diet in this model exhibited the maximum efficacy. In this dosing regimen, the concentration of HCA2969 in plasma was maintained over the EC90 value for lymphocyte homing for 24 h in a day. In contrast, PK analysis of HCA2969 after a single oral administration at 100 mg/kg, revealed that the concentration of HCA2969 in plasma was maintained over the EC90 value for approximately only 8 h. It was also reasonable that twice-daily oral administration of AJM300 at a dose of 100 mg/kg did not achieve significant efficacy in this colitis model (data not shown), since the anti-inflammatory effect may have been counteredacted by lymphocytes infiltrating into the colon.

In this study, peripheral lymphocyte counts dose-dependently increased after oral administration of AJM300 in normal mice. Such an increase was observed in the same dose range as the inhibition of lymphocyte homing. These results suggested that lymphocytes might be forced to remain in the intravascular compartment by the inhibition of α4 integrin-mediated migration and through vascular endothelium. Previous clinical study reported that the increased peripheral lymphocyte counts were also observed with α4 integrin antibody (natalizumab) treatment in clinical trials for Crohn’s disease or ulcerative colitis and could be a reasonable PD marker for blockade of the α4β7 integrin associated with occupancy. Unfortunately, in this colitis model using SCID mice lacking in T and B lymphocytes, it was impossible to use the peripheral lymphocyte count as a PD
marker, because it was too small to detect the size of increase in lymphocyte count. However, the peripheral lymphocyte count might be reasonable for selection of dosing regimens in clinical. Actually, the significant increase in lymphocyte count was observed in human according to the results of the phase II trial of AJM300, which showed that oral administration of AJM300 might be efficacious to the patients with moderately active Crohn’s disease.52 Based on these clinical trials, the optimal dosage is estimated to be 240 mg t.i.d. or above. In addition, a randomized phase II study is currently underway in Japanese patients with moderately active ulcerative colitis.

In conclusion, these results suggest that oral treatment with the small molecule α4 integrin antagonist, AJM300 prevented the development of colitis and that its efficacy was comparable to that of the antibody to α4 integrin. AJM300 might be a promising oral drug for the treatment of IBD.

Conflict of interest statement

Disclosures of financial conflict of interest are summarized below:

- Toshihiko Sugiuira: employee of Ajinomoto Pharmaceuti-
cals Co., Ltd.
- Shunsuke Kageyama: employee of Ajinomoto Pharmaceuti-
cals Co., Ltd.
- Ayatoshi Andou: employee of Ajinomoto Pharmaceuticals 
Co., Ltd.
- Tomoko Miyazawa: employee of Ajinomoto Pharmaceuticals 
Co., Ltd.
- Chieko Ejima: employee of Ajinomoto Pharmaceuticals 
Co., Ltd.
- Akira Nakayama: employee of Ajinomoto Pharmaceuticals 
Co., Ltd.
- Hiroyuki Eda: employee of Ajinomoto Pharmaceuticals 
Co., Ltd.
- Taeko Dohi: nothing to disclose

Acknowledgments

We are grateful to Drs. T. Tanaka, H. Kikutani, and M. Miyasaka (Osaka University, Japan), for providing the cell lines, HEK293, L1-2 and RPMI-8866. We thank the members of the Department of Gastroenterology, Research Institute, International Medical Center of Japan for technical support. We also thank Y. Tanaka, K Kuribayashi, N. Arashida, S. Ueno, I. Fujita, N. Suzuki, S. Nagahisa, Y. Suga, Y. Ikenoue, M. Hashimoto, M. Suzuki, H. Kihara, and M. Murata (Ajinomoto Pharmaceutical Co., Ltd.) for technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.crohns.2013.03.014.

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