A Small Molecule $\alpha_4\beta_1/\alpha_4\beta_7$ Antagonist Differentiates between the Low-Affinity States of $\alpha_4\beta_1$ and $\alpha_4\beta_7$: Characterization of Divalent Cation Dependence

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ABSTRACT

An $\alpha_4\beta_1/\alpha_4\beta_7$ dual antagonist, $^{35}$S-compound 1, was used as a model ligand to study the effect of divalent cations on the activation state and ligand binding properties of $\alpha_4$ integrins. In the presence of 1 mM each Ca$^{2+}$/Mg$^{2+}$, $^{35}$S-compound 1 bound to several cell lines expressing both $\alpha_4\beta_1$ and $\alpha_4\beta_7$, but 2S-(1-benzensulfonyl-pyrrolidine-2S-carbonyl)-amino|4-[4-methyl-2S-(methyl-[2-4-[3-o-toly-ureido]-phenyl])[acyl]-amino] pentanoylamino]-butyric acid (BIO7662), a specific $\alpha_4\beta_1$ antagonist, completely inhibited $^{35}$S-compound 1 binding, suggesting that $\alpha_4\beta_1$ was responsible for the observed binding. $^{35}$S-Compound 1 bound RPMI-8866 cells expressing predominantly $\alpha_4\beta_7$ with a $K_D$ of 1.9 nM in the presence of 1 mM Mn$^{2+}$, and binding was inhibited only 29% by BIO7662, suggesting that the probe is a potent antagonist of activated $\alpha_4\beta_7$. With Ca$^{2+}$/Mg$^{2+}$, $^{35}$S-compound 1 bound Jurkat cells expressing primarily $\alpha_4\beta_1$, with a $K_D$ of 18 nM. In contrast, the binding of $^{35}$S-compound 1 to Mn$^{2+}$-activated Jurkat cells occurred slowly, reaching equilibrium by 60 min, and failed to dissociate within another 60 min. The ability of four $\alpha_4\beta_1/\alpha_4\beta_7$ antagonists to block binding of activated $\alpha_4\beta_1$ or $\alpha_4\beta_7$ to vascular cell adhesion molecule-1 or mucosal addressin cell adhesion molecule-1, respectively, or to $^{35}$S-compound 1 was measured, and a similar rank order of potency was observed for native ligand and probe. Inhibition of $^{35}$S-compound 1 binding to $\alpha_4\beta_1$ in Ca$^{2+}$/Mg$^{2+}$ was used to identify nonselective antagonists among these four. These studies demonstrate that $\alpha_4\beta_1$ and $\alpha_4\beta_7$ have distinct binding properties for the same ligand, and binding parameters are dependent on the state of integrin activation in response to different divalent cations.

Lymphocyte recruitment in the vasculature is regulated by the differential expression and activation of homing receptors (selectins and integrins) on lymphocytes that interact with counter-receptors of the Ig superfamily on endothelial cells. This interaction mediates a multistep process, involving rolling and tethering of leukocytes to endothelial ligands, rapid activation of integrins by locally released chemokines, stable adhesion of activated integrins to endothelial ligands, and transendothelial migration through the vessel wall (Bargatze et al., 1995). Although all integrins expressed on leukocytes can mediate firm adhesion during normal lymphocyte trafficking and in response to inflammatory stimuli, $\alpha_4\beta_1$ and $\alpha_4\beta_7$ are members of a small subset of integrins that can also mediate rolling (Bargatze et al., 1995; Berlin et al., 1995). In vivo studies with monoclonal antibodies or inhibitory peptides demonstrate the pathophysiological role of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ in leukocyte-mediated inflammation in animal models (Foster, 1996; Butcher, 1999), and clinical trials with Antegren (anti-$\alpha_4$) resulted in remission for Crohn's disease patients (Gordon et al., 2001). $\alpha_4$ integrins are constitutively expressed on a variety of leukocytes and can bind to shared or distinct binding partners. $\alpha_4\beta_1$ is expressed on lymphocytes, eosinophils, and monocytes and mediates adhesion to vascular cell adhesion molecule-1 (VCAM-1) expressed on the endothelium and to

ABBRVIATIONS: VCAM-1, vascular cell adhesion molecule-1; MAdCAM-1, mucosal addressin cell adhesion molecule-1; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; NSB, nonspecific binding; FACS, fluorescence-activated cell sorting; CS-1, connecting segment-1; mAb, monoclonal antibody; cmpd, compound.
the connecting segment-1 (CS-1) subdomain of human fibronectin in the extracellular matrix. \( \alpha_{4}\beta_1 \) and \( \alpha_4\beta_7 \) are coexpressed on peripheral blood leukocytes, and \( \alpha_4\beta_7 \) is highly expressed on a discrete subpopulation of gut-homing memory T and B lymphocytes, mediating lymphocyte adhesion within the vasculature of the gastrointestinal tract, where its major ligand, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), is preferentially expressed on high endothelial venules (Butcher, 1999). Although both \( \alpha_4\beta_7 \) and \( \alpha_4\beta_1 \) can bind VCAM-1 and CS-1, \( \alpha_4\beta_1 \) does not bind MAdCAM-1, and \( \alpha_4\beta_7 \) binds to MAdCAM-1 with higher affinity than to VCAM-1 or to CS-1 (Berlin et al., 1993).

Key motifs for the binding of \( \alpha_4\beta_7 \) and \( \alpha_4\beta_1 \) to native ligands have been defined as leucine-aspartic acid-threonine in MAdCAM-1 (Viney et al., 1996), isoleucine-aspartic acid-serine in VCAM-1 (Wang et al., 1995), and leucin-aspartic acid-valine in CS-1 (Wayner and Kovach, 1992). Small molecule antagonists of \( \alpha_4\beta_1 \) that mimic the LDT motif have been described that block the binding of \( \alpha_4\beta_7 \)-expressing cells to MAdCAM-Ig in the presence of Mn\(^{2+} \) (Carson et al., 1997; Shroff et al., 1998; Martin et al., 1999; Harriman et al., 2000; Egger et al., 2002). Similarly, antagonists of \( \alpha_4\beta_7 \) have been reported to block binding of Mn\(^{2+} \)-activated (Jackson et al., 1997; Vanderslice et al., 1997; Lin et al., 1998; Hagmann et al., 2001; Muller et al., 2001) and unactivated \( \alpha_4\beta_1 \) (Chen et al., 1999, 2001) to ligand in vitro.

The essential role of cation-binding sites in regulating integrin function is known, but the coordination of each cation-binding site and the individual role of different metal cations is not well understood (Leitinger et al., 2000). All integrin \( \alpha \)-subunits have seven homologous 60-amino acid repeats at the N terminus that have been predicted to fold into a propeller structure (Shimaoka et al., 2002), and three to four of these repeats are thought to be involved in the structural role in coordinating the binding of ligand to the I-domain containing integrins. Although \( \alpha_4 \) does not contain an I-domain, an I-like domain that contains a metal ion-dependent activation site-like motif is present in the \( \beta \)-chain of all integrins. Although the effect of divalent cations on \( \alpha_4\beta_7 \)-ligand interactions has not been extensively characterized, recent studies have shown that Ca\(^{2+} \) is essential to support rolling under shear flow, whereas Mg\(^{2+} \) can promote firm adhesion of cells expressing \( \alpha_4\beta_7 \) to MAdCAM-1 (de Chateau et al., 2001).

To assess the effect of divalent cations on the activation state of \( \alpha_4 \) integrins expressed on human lymphocytes, we used a novel dual \( \alpha_4\beta_3/\alpha_4\beta_7 \) antagonist, \(^{35}\)S-compound 1 (Fig. 1), as a model ligand. A similar approach has been used to study multiple activation states of \( \alpha_4\beta_7 \) through their different affinities for a small molecule ligand (Chen et al., 1999, 2001), but the binding of a small molecule ligand to different activation states of \( \alpha_4\beta_3 \) has not been described. These studies provide new information that \( \alpha_4\beta_3 \) and \( \alpha_4\beta_7 \) have distinct binding affinities for the same small molecule ligand, and binding is dependent on the state of integrin activation in response to different divalent cations.

**Materials and Methods**

**Compounds.** Compound 1, \( N-(N\text{-benzenesulfonyl}-4(R)\text{-cycloproplylamin}-2(S)-prolyl)-L\text{-arginine} \), is a 7 bind antagonist, \(^{35}\)S-compound 1 (Fig. 1), as a model ligand. A similar approach has been used to study multiple activation states of \( \alpha_4\beta_7 \) through their different affinities for a small molecule ligand (Chen et al., 1999, 2001), but the binding of a small molecule ligand to different activation states of \( \alpha_4\beta_3 \) has not been described. These studies provide new information that \( \alpha_4\beta_3 \) and \( \alpha_4\beta_7 \) have distinct binding affinities for the same small molecule ligand, and binding is dependent on the state of integrin activation in response to different divalent cations.

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**Antibodies and Cell Lines.** The following purified monoclonal antibodies were obtained from BD PharMingen (San Diego, CA): 4B4 (mouse anti-human \( \beta_1 \)), FIB27 (rat anti-mouse \( \beta_3 \), which cross-reacts with human \( \beta_3 \)), and isotype controls (mouse IgG1, rat IgG2b). HP2/1 (mouse anti-human \( \alpha_4 \)) was obtained from Coulter/Immunotech (Hialeah, FL). The following cell lines were used: RPMI-8866 cells (human B cell line) obtained from John A. Wilkins (University of
Dual Antagonist Binds Unactivated $\alpha_2\beta_1$ and Activated $\alpha_2\beta_7$

### Results

**Potency of Compound 1 in $\alpha_2\beta_1$ and $\alpha_2\beta_7$ Ligand Binding Assays.** Compound 1 (Fig. 1) represents one of a structural class of potent $\alpha_2\beta_1$ antagonists (Hagmann et al., 2001). To determine whether compound 1 could also block ligand binding to $\alpha_2\beta_7$, the ability of this compound to inhibit binding of $^{35}$S-MadCAM-Ig to RPMI-8866 cells in the presence of the divalent cation $\text{Mn}^{2+}$ was evaluated using methods described previously (Egger et al., 2002). RPMI-8866 cells, a human B cell line, were chosen for the assay, because they express high levels of $\alpha_2\beta_7$ receptors/cell, but low levels of $\alpha_2\beta_7$ receptors/cell, as demonstrated by quantitative flow cytometry (Fig. 2A). In addition, the specificity of MadCAM-Ig binding to $\alpha_2\beta_7$ on the RPMI-8866 cells has been confirmed by demonstrating that...
anti-α and anti-β mAbs block binding, whereas an anti-β mAb does not block binding (Egger et al., 2002). Furthermore, the low levels of αβ expressed on the RPMI-8666 cell line do not bind VCAM-Ig in the presence of anti-β mAbs and 1 mM Mn²⁺ or 1 mM Ca²⁺/Mg²⁺ (data not shown). Thus, RPMI-8666 cells can be used to evaluate the potency of compounds in blocking binding of αβ, but not αβ, to MadCam-Ig. Compound 1 inhibited the binding of MadCam-Ig to αβ with an IC₅₀ of 1.1 nM (Table 1).

To compare the potency of compound 1 for blockade of αβ under similar conditions, the ability of the compound to inhibit ¹²⁵I-VCA-M-Ig binding to Jurkat cells expressing αβ, in the presence of the divalent cation Mn²⁺ was performed as described previously (Egger et al., 2002). For this assay, Jurkat cells, a human T cell line, were chosen, because they express high levels of αβ (≈90,000 αβ receptors/cell), but low levels of αβ (≈7,000 αβ receptors/cell) (Fig. 2B). Specificity of VCAM-Ig binding to αβ on Jurkat cells, was confirmed previously using anti-α and anti-β mAbs to completely abrogate binding, in the absence of inhibition by anti-β mAb (Egger et al., 2002). The low level of αβ expressed on the Jurkat cell line does not bind MadCam-Ig in the presence of 1 mM Mn²⁺ or 1 mM Ca²⁺/Mg²⁺ (data not shown), indicating that Jurkat cells can be used to evaluate the potency of compounds in blocking binding of αβ, but not αβ, to VCAM-Ig. Compound 1 inhibited the binding of VCAM-Ig to αβ with an IC₅₀ of 0.10 nM (Table 1). Thus, compound 1 is a potent dual αβ/αβ antagonist.

Divalent Cation-Dependent Binding of a Radiolabeled αβ/αβ Antagonist Probe, ³⁸S-Compound 1, to RPMI-8666 Cells, K562/αβ Cells, and HUT-78 Cells. αβ-ligand interactions are dependent on divalent cations, which modulate the affinity state of the integrins for their ligands (Leitinger et al., 2000). To test the divalent cation dependence of binding, an assay was developed to measure the binding of ³⁸S-compound 1 to RPMI-8666 cells expressing activated or unactivated αβ, in the presence of the divalent cations Mn²⁺ or Ca²⁺/Mg²⁺, respectively (Fig. 3). In the presence of 1 mM Mn²⁺, RPMI-8666 cells bound ³⁸S-compound 1 with a ratio of specific to nonspecific binding of 12 (Fig. 3A). In the presence of 1 mM Ca²⁺/Mg²⁺, ³⁸S-compound 1 bound RPMI-8666 cells with a ratio of specific to nonspecific binding of 6. Specific binding was not observed in the presence of either 1 mM Ca²⁺ or 1 mM Mg²⁺ alone (data not shown). The inclusion of 10 mM EDTA in the binding reaction abrogated specific binding, as expected.

To determine whether the low level of αβ present on RPMI-8666 cells (Fig. 2A) contributed to the observed binding of ³⁸S-compound 1, binding was measured with or without 100 nM BIO7662 added to specifically block αβ (Fig. 3A). BIO7662, a highly selective inhibitor of αβ (Chen et al., 2001; Pepinsky et al., 2002; Leone et al., 2003), was used for these studies because none of the available anti-α or anti-β neutralizing monoclonal antibodies blocked the binding of ³⁸S-compound 1 to αβ even at concentrations up to 3.3 μg/ml (data not shown). BIO7662 (100 nM) was selected to completely saturate αβ (IC₅₀ of 0.03 nM in the αβ/³⁸S-VCA-M-Ig binding assay; Table 1) without interfering with αβ (IC₅₀ of 3.34 μM in the αβ/³⁸S-MADCAM-Ig binding assay; Table 1). In the presence of 1 mM Mn²⁺ and 100 nM BIO7662, RPMI-8666 cells bound ³⁸S-compound 1 (input

![Fig. 2. Surface expression of αβ and αβ on RPMI-8666 and Jurkat cell lines. Quantitative FACS analysis was used to determine the density of antibody binding sites for αβ and αβ expressed on the surface of the RPMI-8666 human B cell line (A) and the Jurkat human T cell line (B) as described under Materials and Methods. The data represent the mean number of receptors expressed per cell (receptor) for at least two independent experiments for each cell type. The isotype control, anti-α, anti-β, and anti-β mAb-treated cell profiles are represented by the gray, black, dashed, and dotted lines, respectively.](image-url)

### Table 1

<table>
<thead>
<tr>
<th>Activity of antagonists of αβ/αβ in ligand binding assays</th>
<th>Binding Assay</th>
<th>IC₅₀ (nM) or % Inhibition</th>
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<td>Compounds were tested using a 10-point titration in assays that measure the binding of either ¹²⁵I-MADCAM-Ig, ¹²⁵I-VCAM-Ig, or ³⁸S-compound 1 to cells expressing αβ to determine compound potency and specificity. Assays for activated αβ measured the binding of either ¹²⁵I-MADCAM-Ig to RPMI-8666 cells in the presence of 1 mM Mn²⁺ or the binding of ³⁸S-compound 1 to RPMI-8666 cells in the presence of 1 mM Mn²⁺ and 100 nM BIO7662. Similarly, assays for activated αβ measured the binding of either ¹²⁵I-VCAM-Ig or ³⁸S-compound 1 to Jurkat cells in the presence of 1 mM Mn²⁺, whereas unactivated αβ measured the binding of ³⁸S-compound 1 to Jurkat cells in the presence of 1 mM Ca²⁺/Mg²⁺. Values represent the average of at least two independent experiments, and all values were within 95% confidence limits.</td>
<td><strong>Cmpd 1</strong></td>
<td><strong>Cmpd 2</strong></td>
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<td>----------------------------------------------------------</td>
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</tr>
<tr>
<td>αβ (RPMI-8666 cells)</td>
<td>¹²⁵I-MADCAM-Ig/Mn²⁺</td>
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</tr>
<tr>
<td>¹²⁵I-Cmpd 1/Mn²⁺</td>
<td>0.4</td>
<td>65.0</td>
</tr>
<tr>
<td>αβ (Jurkat cells)</td>
<td>¹²⁵I-VCAM-Ig/Mn²⁺</td>
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</tr>
<tr>
<td>¹²⁵I-Cmpd 1/Mn²⁺</td>
<td>2.5</td>
<td>6.3</td>
</tr>
<tr>
<td>¹²⁵I-Cmpd 1/Mn²⁺</td>
<td>2.3</td>
<td>6.0</td>
</tr>
</tbody>
</table>

N.D., not determined.
BIO7662 was not significantly different from background binding, suggesting that low levels of unactivated $\alpha_\beta_7$ expressed on RPMI-8866 cells are responsible for the binding of $^{35}$S-compound 1 under these conditions. Comparison of the specific binding of $^{35}$S-compound 1 to RPMI-8866 cells measured in the presence of 1 mM Ca$^{2+}$/Mg$^{2+}$ with or without BIO7662 was significantly different with a $P$ value $< 0.01$. Furthermore, when incubating RPMI-8866 cells with 0 to 30 nM $^{35}$S-compound 1 in the presence of 1 mM Ca$^{2+}$/Mg$^{2+}$ and 100 nM BIO7662, no significant binding above background was observed (data not shown). The results shown in Fig. 3A indicate that $\alpha_\beta_7$ on RPMI-8866 cells requires Mn$^{2+}$ to support binding of $^{35}$S-compound 1 and that the unactivated state of the receptor does not support binding.

To determine whether the inability of $^{35}$S-compound 1 to bind unactivated $\alpha_\beta_7$ was dependent on cell type, we measured binding to K562 cells stably transfected with $\alpha_4\beta_7$ (K562/$\alpha_4\beta_7$) in the presence of different divalent cations. As demonstrated by quantitative FACS analysis, K562/$\alpha_4\beta_7$ cells express ~200,000 copies/cell of $\alpha_\beta_1$ and ~200,000 copies/cell of $\alpha_\beta_7$ (data not shown). In the presence of 1 mM Mn$^{2+}$ and 100 nM BIO7662, K562/$\alpha_4\beta_7$ cells bound $^{35}$S-compound 1 with a 19-fold ratio of specific to nonspecific binding, and binding was reduced by 63% compared with cells not treated with BIO7662 (Fig. 3B). Thus, the binding of $^{35}$S-compound 1 to activated K562/$\alpha_\beta_7$ cells is mediated by both $\alpha_\beta_1$ and $\alpha_\beta_7$, with each integrin having a similar contribution to the total binding. In the presence of 1 mM Ca$^{2+}$/Mg$^{2+}$, $^{35}$S-compound 1 bound K562/$\alpha_\beta_7$ cells with a ratio of specific to nonspecific binding of 31-fold (Fig. 3B), but pretreating the cells with 100 nM BIO7662 abrogated binding (Fig. 3B), indicating that K562/$\alpha_4\beta_7$ cell binding to $^{35}$S-compound 1 is mediated solely by unactivated $\alpha_\beta_1$. To extend the above-mentioned findings to another cell line, the effect of divalent cations on the binding of $^{35}$S-compound 1 to HUT-78 cells was evaluated. By FACS analysis, HUT-78 cells express equal proportions of both $\alpha_\beta_1$ and $\alpha_\beta_7$ (data not shown), and the relative receptor density of $\alpha_\beta_7$ was similar to levels observed on RPMI-8866 cells (Erle et al., 1994). Mn$^{2+}$-activated HUT-78 cells bound to $^{35}$S-compound 1 with a 10- or 2-fold ratio of specific to nonspecific binding in the absence or presence of 100 nM BIO7662, respectively (Fig. 3C). In the presence of 1 mM Ca$^{2+}$/Mg$^{2+}$, $^{35}$S-compound 1 bound unactivated HUT-78 cells with a ratio of specific to nonspecific binding of 9-fold, but failed to bind unactivated cells after pretreatment with 100 nM BIO7662 (Fig. 3C), indicating that unactivated $\alpha_\beta_1$ is also completely responsible for the binding of $^{35}$S-compound 1 to HUT-78 cell binding to $^{35}$S-compound 1. Thus, the binding properties of $^{35}$S-compound 1 for different activation states of $\alpha_\beta_7$ are not dependent on cell type or expression levels of $\alpha_\beta_1$ relative to $\alpha_\beta_7$, but rather are dependent on the activation state of the integrins.

**Binding Kinetics of $^{35}$S-Compound 1 to RPMI-8866 Cells.** After three cell lines expressing both $\alpha_\beta_1$ and $\alpha_\beta_7$ were evaluated, subsequent studies on $\alpha_\beta_7$ focused on characterizing the binding of $^{35}$S-compound 1 to RPMI-8866 cells that predominantly express $\alpha_\beta_7$. To determine whether an assay could be developed to study both the association and dissociation of unlabeled compounds from binding to activated $\alpha_\beta_7$, equilibrium and kinetic studies for the binding of $^{35}$S-compound 1 to $\alpha_\beta_7$ were performed in the presence of Mn$^{2+}$ with cells pretreated with 100 nM BIO7662 (Fig. 4).
Fig. 4. Equilibrium binding of $^{35}$S-compound 1 to RPMI-8866 cells in the presence of Mn$^{2+}$. RPMI-8866 cells were incubated with increasing concentrations of $^{35}$S-compound 1 (0–30 nM) in binding buffer containing 1 mM Mn$^{2+}$ and 100 nM BIO7662. Specific binding (cpm) was measured as described under Materials and Methods, and nonspecific binding was determined in the presence of 1 μM unlabeled compound 1. $B_{\text{max}}$ values (receptors/cell) were calculated by nonlinear regression analysis by using a one-site binding model ($R^2$ value of 0.98). The data shown are representative of at least two independent experiments containing duplicate samples in each experiment.

Fig. 5. On and off rates for $^{35}$S-compound 1 binding to RPMI-8866 cells in the presence of Mn$^{2+}$. RPMI-8866 cells were incubated for the indicated times with 6.5 nM $^{35}$S-compound 1 and 5 nM unlabeled compound 1 in binding buffer containing 1 mM Mn$^{2+}$ and 100 nM BIO7662. Specific binding (cpm) for compound association (•) or dissociation (▲) was measured as described under Materials and Methods, with nonspecific binding determined in the presence of 1 μM unlabeled compound 1. $k_{\text{on}}$ was determined at a ligand concentration of 11.5 nM by fitting the data to a one-phase exponential association equation ($R^2$ value of 0.92). The indicated on rate ($k_{\text{on}}$), off rate ($k_{\text{off}}$) and $K_d$ for the binding of $^{35}$S-compound 1 to αβ7 on RPMI-8866 cells were determined from the kinetic measurements by fitting the data to a one-phase exponential association or decay equation ($R^2$ value of 0.99 for each curve). The data shown are representative of at least two independent experiments containing duplicate samples in each experiment.

To determine whether Ca$^{2+}$ can affect the ability of Mg$^{2+}$ or Mn$^{2+}$ to activate αβ7, RPMI-8866 cells were treated with 100 nM BIO7662 and increasing concentrations of Ca$^{2+}$ (0.04–5 mM) in combination with 5 mM Mn$^{2+}$ or 5 mM Mg$^{2+}$, to achieve maximal activation and partial activation, respectively. In the presence of 5 mM Mn$^{2+}$, Ca$^{2+}$ at 1.25 mM and 5 mM inhibited the binding of $^{35}$S-compound 1 to RPMI-8866 cells by 35 and 65%, respectively (Fig. 6B). Similarly, when combined with 5 mM Mg$^{2+}$, Ca$^{2+}$ at 1.25 and 5 mM inhibited the binding of $^{35}$S-compound 1 to RPMI-8866 cells by 38 and 51%, respectively.

To further explore the interaction between Mn$^{2+}$ and Ca$^{2+}$, the binding of $^{35}$S-compound 1 to αβ7 was measured in the presence of increasing concentrations of Ca$^{2+}$ (0.1–100 mM) in combination with three different fixed concentrations of Mn$^{2+}$ (0.2, 1, and 5 mM). Ca$^{2+}$ inhibited the binding of $^{35}$S-compound 1 to RPMI-8866 cells with IC$_{50}$ values of 1.0, 4.0, and 14.0 mM, respectively. A double reciprocal plot of these data (Fig. 6D) indicated that the inhibition observed by Ca$^{2+}$ in the presence of Mn$^{2+}$ is noncompetitive in nature, and this lack of competition is between Ca$^{2+}$ and Mn$^{2+}$ rather than ligand.

**Divalent Cation-Dependent Binding $^{35}$S-Compound 1 to Jurkat Cells.** To understand how divalent cations regulate the activation state of αβ7, an assay was developed to measure the binding of $^{35}$S-compound 1 to Jurkat cells expressing activated or unactivated αβ7 in the presence of the divalent cations Mn$^{2+}$ or Ca$^{2+}$/Mg$^{2+}$, respectively (Fig. 7). Binding of $^{35}$S-compound 1 to Jurkat cells was dose-dependent and reached saturation at 5 nM. Because available anti-αβ7 mAb did not compete with the binding of $^{35}$S-compound 1, the specificity of $^{35}$S-compound 1 binding to
was determined by direct competition with unlabeled compound 1 at 1 μM. Specific counts bound at saturation provided a direct measure of 4 integrin expression levels, and, based on this value, Jurkat cells used in these studies have approximately 64,000 copies (in Mn²⁺) and 57,000 copies (in Ca²⁺/Mg²⁺) of 4 integrin/cell, which is in agreement with the receptor density determined by quantitative FACS analysis (Fig. 2B). Binding was dependent on the presence of divalent cations, and was blocked in the presence of 10 mM EDTA (data not shown).

Fig. 6. Binding of ³⁵S-compound 1 to RPMI-8866 cells under different divalent cation conditions. For all experiments, RPMI-8866 cells were pretreated with 100 nM BIO7662 and incubated at room temperature for 45 min with 110 pM of ³⁵S-compound 1. A, cells were incubated in the presence of increasing concentrations of Mn²⁺ (●) or Mg²⁺ (□). Specific binding (cpm) was measured as described under Materials and Methods, and nonspecific binding was determined in the presence of 1 μM unlabeled compound 1. B, cells were incubated in the presence of 5 mM Mn²⁺ (●) or 5 mM Mg²⁺ (□) and increasing concentrations of Ca²⁺, and specific counts bound were determined as described above. C, cells were incubated in the presence of 0.2 mM Mn²⁺ (●), 1 mM Mn²⁺ (■), or 5 mM Mn²⁺ (□) and increasing concentrations of Ca²⁺. D, a double reciprocal plot of the data shown in C. By linear regression analysis, the three lines intersect approximately on the x-axis, indicative of noncompetitive inhibition. R² values are 0.97 (5 mM Mn²⁺ with Ca²⁺), 0.99 (1 mM Mn²⁺ with Ca²⁺) and 0.99 (0.2 mM Mn²⁺ with Ca²⁺). All data shown are representative of at least two independent experiments containing duplicate samples in each experiment.

α₄β₇ was determined by direct competition with unlabeled compound 1 at 1 μM. Specific counts bound at saturation provided a direct measure of α₄β₁ expression levels, and, based on this value, Jurkat cells used in these studies have approximately 64,000 copies (in Mn²⁺) and 57,000 copies (in Ca²⁺/Mg²⁺) of α₄β₂ integrin/cell, which is in agreement with the receptor density determined by quantitative FACS analysis (Fig. 2B). Binding was dependent on the presence of divalent cations, and was blocked in the presence of 10 mM EDTA (data not shown).

As observed for the binding of ³⁵S-compound 1 to α₄β₇, the apparent Kₐ for binding seemed to be small compared with the concentration of α₄β₁ (350 and 320 pM, respectively) present in the binding assay (Fig. 7). The kinetic assessment of binding of compound 1 to α₄β₁ in the presence of 1 mM Mn²⁺ indicated that binding occurred very slowly, reaching equilibrium by 60 min, and dissociation was not observed after another 60 min of incubation (Fig. 8A), or even after another 180 min (48,036 specific cpm bound after 240 min of total incubation time; data not shown). In the presence of 1 mM Ca²⁺/Mg²⁺, binding occurred with a kₘ of 0.01 nM⁻¹ min⁻¹, reaching equilibrium within 10 min, and dissociation was rapid, with a kₐ of 0.24 min⁻¹, resulting in a Kₐ of 18 nM (Fig. 8B). Because the saturation binding curves measured titration of the receptor to full occupancy, the Kₐ values obtained from the kinetic binding curves represent the actual binding affinity. Although binding of the protein ligand ¹²⁵I-VCAM-Ig requires an activated state of 4 integrin, achieved by adding 1 mM Mn²⁺, ³⁵S-compound 1 is observed to bind both activated and unactivated states of α₄β₁. Although similar association rates were observed for the binding of ³⁵S-compound 1 to unactivated and activated α₄β₁, dissociation rates were dependent on the activation state of the integrin, with dissociation from the activated receptor indiscernible under the conditions of the assay.

Ability of Antagonists of α₄β₁ and α₄β₇ to Block the Binding of Native Ligand or ³⁵S-Compound 1 to RPMI-8866 or Jurkat Cells. After demonstrating that ³⁵S-compound 1 can be used to analyze α₄ interactions on both RPMI-8866 and Jurkat cell lines, we were interested in determining the potency of four α₄β₁/α₄β₇ antagonists. Compounds were initially evaluated for their ability to block ¹²⁵I-MAdCAM-Ig
binding to RPMI-8866 cells and 125I-VCAM-Ig binding to Jurkat cells, in the presence of Mn2+ or Ca2+/Mg2+. Jurkat cells were incubated with increasing concentrations of 35S-compound 1 (0–54 nM) in binding buffer containing 1 mM Mn2+ (A) or 1 mM Ca2+/Mg2+ (B) and 100 nM BIO7662. Specific binding (cpm) was measured as described under Materials and Methods, with nonspecific binding determined in the presence of 1 μM unlabeled compound 1. Bmax values (receptors/cell) were calculated by nonlinear regression analysis by fitting the data to a one-site binding model (R2 value of 0.95 or 0.97 for A and B, respectively). The data shown are representative of at least two independent experiments.

Fig. 7. Equilibrium binding of 35S-compound 1 to Jurkat cells expressing α4β1 in the presence of Mn2+ or Ca2+/Mg2+. Jurkat cells were incubated for the indicated times with 6.5 nM 35S-compound 1 and 5 nM unlabeled compound 1 in binding buffer containing 1 mM Mn2+ or Ca2+/Mg2+. A, specific binding (cpm) for compound association (□) to or dissociation (▲) from Mn2+-activated α4β1 was measured as described under Materials and Methods. B, specific binding (cpm) for compound association (□) to or dissociation (▲) from unactivated α4β1 was measured as outlined above and as described under Materials and Methods. kobs was determined at a ligand concentration of 11.5 nM, and the on rate (k+), off rate (k-), and K values for the binding of 35S-compound 1 were determined from kinetic measurements by fitting the data to a one-phase exponential association or decay equation (R2 value of 0.86 and 0.99 for association and dissociation, respectively). The data shown are representative of at least two independent experiments containing duplicate values in each experiment.

Fig. 8. On and off rates for 35S-compound 1 binding to Jurkat cells in the presence of Mn2+ or Ca2+/Mg2+. A, Jurkat cells were incubated for the indicated times with 6.5 nM 35S-compound 1 and 5 nM unlabeled compound 1 in binding buffer containing 1 mM Mn2+ or Ca2+/Mg2+. B, specific binding (cpm) for compound association (□) to or dissociation (▲) from Mn2+-activated α4β1 was measured as described under Materials and Methods. B, specific binding (cpm) for compound association (□) to or dissociation (▲) from unactivated α4β1 was measured as outlined above and as described under Materials and Methods. kobs was determined at a ligand concentration of 11.5 nM, and the on rate (k+), off rate (k-), and K values for the binding of 35S-compound 1 were determined from kinetic measurements by fitting the data to a one-phase exponential association or decay equation (R2 value of 0.86 and 0.99 for association and dissociation, respectively). The data shown are representative of at least two independent experiments containing duplicate values in each experiment.
the presence of Mn$^{2+}$ was also measured. The concentration of $^{35}$S-compound 1 used for the binding assay was maintained at less than 150 pM, based on an IC$_{50}$ of 2.5 and 2.3 nM for competition by unlabeled compound 1 with activated and unactivated Jurkat cells, respectively (Table 1), and the potency of compounds tested is shown in Table 1. Despite the overall shift toward reduced potency (26- to 79-fold less potent) in the $^{35}$S-compound 1/activated Mg$^{2+}$ potency for activated and unactivated

Nonselective compounds, those that have relatively equal potency in the presence of Ca$^{2+}$ and Mg$^{2+}$, respectively, but only bound activated $\alpha_4\beta_7$. Binding kinetics revealed that $^{35}$S-compound 1 dissociated from activated $\alpha_4\beta_7$ and unactivated $\alpha_4\beta_7$, but failed to dissociate from activated $\alpha_4\beta_7$, in the time frame observed. Although radiolabeled probes have been used to study the function of $\alpha_4\beta_7$-ligand interactions (Chen et al., 1999, 2001), this is the first report that a dual $\alpha_4\beta_7$/$\alpha_4\beta_7$ antagonist can be used to elucidate the effect of divergent cations on both $\alpha_4\beta_7$- and $\alpha_4\beta_7$-ligand interactions, defining distinct binding properties of the probe for each integrin.

Integrins are known to exist in multiple affinity states in the presence of different divalent cations (Shimaoka et al., 2002). For many integrin-ligand interactions, including those of $\beta_2$, $\beta_3$, and $\beta_7$ integrins, the most efficient binding to immobilized ligand occurs in the presence of Mn$^{2+}$, with less binding in the presence of Mg$^{2+}$, and little or no binding in the presence of Ca$^{2+}$ (Dransfield et al., 1992; Mould et al., 1995; Chen et al., 1999; Chigaev et al., 2001). Binding of cells expressing $\alpha_4\beta_7$ to E-cadherin is observed with 1 mM Mn$^{2+}$/Mg$^{2+}$/Ca$^{2+}$ or with EGTA plus 10 mM Mg$^{2+}$, whereas negligible binding occurs in the presence of 1 mM Ca$^{2+}$/Mg$^{2+}$ (Higgins et al., 1998). These published results are consistent with observations in this report where an 8-fold difference in affinity was detected between the binding of $^{35}$S-compound 1 to Mn$^{2+}$-activated $\alpha_4\beta_7$ and $\alpha_4\beta_7$ in the presence of Ca$^{2+}$/Mg$^{2+}$ (Fig. 3A with BIO7662). Mn$^{2+}$ enhanced the binding of $^{35}$S-compound 1 to $\alpha_4\beta_7$ with an EC$_{50}$ of 0.5 mM (Fig. 6A), whereas 1 mM Ca$^{2+}$/Mg$^{2+}$ supported binding to $\alpha_4\beta_7$, but not $\alpha_4\beta_7$ (Figs. 3A and 7B). Ca$^{2+}$ alone did not support binding to $\alpha_4\beta_7$, and nonphysiological levels of Mg$^{2+}$ were required to stimulate binding to $\alpha_4\beta_7$ (Fig. 6A).

The 29% reduction in the binding of compound 1 to RPMI-8866 cells in Mn$^{2+}$ with 100 nM BIO7662 (Fig. 3A) added was surprising, because only a 5 to 10% drop in total counts bound was expected due to the binding to $\alpha_4\beta_7$ (Fig. 2A). One explanation for this finding is that with 100 nM BIO7662 added, a small fraction of the $\alpha_4\beta_7$ was occupied by BIO7662 and blocked binding of $^{35}$S-compound 1. To test this possibility, we reevaluated the binding of BIO7662 to $\alpha_4\beta_7$, but on JY cells, a human B cell line, which expresses high levels of $\alpha_4\beta_7$, and no detectable $\alpha_4\beta_1$. First, binding was evaluated using radiolabeled BIO7662. No specific measurable binding was observed with 10 nM $^{35}$S-BIO7662, supporting the observation that the affinity of BIO7662 for $\alpha_4\beta_7$ is low. Second, the IC$_{50}$ of BIO7662 for $\alpha_4\beta_7$ was measured in an adhesion format in which the ability of BIO7662 to block binding of fluorescently labeled JY cells to plates coated with N-[4-(6-aminohexane-1-sulfonylamino)-2,6-dichlorobenzoyl]-4-(2,6-dimethoxyphenyl)-1-phenylalanine trifluoroacetate-bovine serum albumin conjugate was quantified. N-[4-(6-Aminohexane-1-sulfonylamino)-2,6-dichlorobenzoyl]-4-(2,6-dimethoxyphenyl)-1-phenylalanine trifluoroacetate (Pepinsky et al., 2002), an analog of compound 1 that had been engineered to contain a linker for cross-linking, was used for these studies. IC$_{50}$ values in this assay of 10 nM in 1 mM Ca$^{2+}$/Mg$^{2+}$ and 1 mM Mn$^{2+}$ were observed to confirm that BIO7662 is a poor inhibitor of $\alpha_4\beta_7$. Although these studies support the integrin selectivity data for BIO7662, the separation under activating conditions between the IC$_{50}$ of BIO7662 in the adhesion assay (1 mM) and the amount added to prevent $\alpha_4\beta_7$ binding, 100 nM, was not as great as was predicted from the MAdCAM-Ig binding study (Table 1), and consequently binding of BIO7662 to $\alpha_4\beta_7$ is likely to have accounted for the reduction in the counts bound for $^{35}$S-compound 1.

The RPMI-8866 cell line has been widely used in binding assays to identify $\alpha_4\beta_7$ antagonists (Carson et al., 1997; Shroff et al., 1998; Martin et al., 1999; Harriman et al., 2000). We provide the first evidence that low levels of unactivated $\alpha_4\beta_7$ expressed on RPMI-8866 cells are capable of binding to a small molecule antagonist, such as $^{35}$S-compound 1, and we demonstrate that unactivated $\alpha_4\beta_7$ on a variety of cell lines does not bind $^{35}$S-compound 1. Low levels of $\alpha_4\beta_7$ expressed on RPMI-8866 cells do not contribute to binding of MAdCAM-Ig, and the binding of MAdCAM-1 to cells in suspension expressing $\alpha_4\beta_7$ is known to require Mn$^{2+}$ (Egger et al., 2002). Blockade of $\alpha_4\beta_7$ by BIO7662 abrogated binding of $^{35}$S-compound 1 to RPMI-8866, K562/$\alpha_4\beta_7$, or HUT-78 cells in Ca$^{2+}$/Mg$^{2+}$, indicating that $\alpha_4\beta_7$ was responsible for binding observed in the unactivated condition (Fig. 3). Thus, the binding properties of $\alpha_4$ integrins coexpressed on the same cell varies depending on the state of activation.

Recent reports have described the use of radiolabeled small molecule ligands that can be used to study $\alpha_4\beta_7$-ligand interactions (Chen et al., 1999, 2001). For example, $^{35}$S-BIO7662 is a specific $\alpha_4\beta_7$ antagonist that binds with high affinity ($K_d < 10$ pM) to both unactivated and activated $\alpha_4\beta_7$ (Chen et al., 2001). Solution binding studies on purified $\alpha_4\beta_7$ identified a high-affinity site for Ca$^{2+}$ that stimulates BIO7662 binding and a low-affinity site that functions independently of
BIO7662 binding (Chen et al., 2001). Similarly, [3H]BIO1211 has been identified as a specific αβ₁ antagonist that binds with low affinity (Kᵦᵦ of 20–40 nM) to unactivated αβ₁, but with high affinity to activated αβ₁ (Kᵦᵦ of 18–100 pM) to Chen et al. (1999). Studies with antagonists of αβ₁ demonstrate that the metal ion dependence of ligand binding is affected by the affinity of the ligand for αβ₁, because the EDT₄₀ concentrations required to support BIO7662 binding were 2-fold lower for Mn²⁺, -fold lower for Mg²⁺, and >1000-fold lower for Ca²⁺, compared with the concentrations required to support BIO1211 binding (Chen et al., 1999, 2001).

Compared with association rates reported for BIO7662, BIO1211 and an LDV-based peptide, the association rate for compound 1 binding to αβ₁ in the presence of 1 mM Ca²⁺/Mg²⁺ was greater by an order of magnitude (Chen et al., 1999, 2001; Chigaev et al., 2001). Consistent with published reports on the binding properties of specific αβ₁ antagonists, ⁴²⁵⁸S-compound 1 had similar association rates for binding to unactivated and activated αβ₁, but dissociation rates were highly dependent on the state of activation (Fig. 8). ⁴²⁵⁸S-Compounded 1 rapidly dissociated from Jurkat cells expressing unactivated αβ₁; but dissociation from activated αβ₁ could not be observed even after 180 min. Although ⁴²⁵⁸S-compound 1 is a lower affinity ligand than BIO1211 or BIO7662, this is the first report of a dual αβ₁/αβ₂ antagonist that can be used to study the metal-cation dependence of both αβ₁ and αβ₂ binding.

Finally, observations presented here have key implications for the development of a dual αβ₁/αβ₂ antagonist that is selective or nonselective for αβ₁. Both αβ₁ and αβ₂ are pharmaceutical targets for a variety of inflammatory disorders, including asthma, inflammatory bowel disease, and multiple sclerosis (Gordon et al., 2001; Jackson, 2002). The two α₂ integrins are unique in their ability to act both as low-affinity receptors that participate in rolling and tethering, and as high-affinity receptors that mediate firm adhesion (Bargatze et al., 1995; Berlin et al., 1995). A continuum of integrin activation states is known to exist in vivo, and the Mn²⁺-activated state may not resemble the highest affinity state that occurs under physiological conditions. Integrin activation through G protein-linked receptors occurs over several seconds, whereas activation-dependent arrest takes minutes (Butcher, 1999). Because the association rate of an antagonist may be slower than the rate of integrin activation, a nonselective antagonist may be required for effective blockade of ligand binding, and nonselective neutralizing antibodies against α₂ and αβ₂ are known to be efficacious in vivo models (Foster, 1996; Butcher, 1999). Both a selective antagonist that blocks to the activated state or a nonselective antagonist will inhibit the extravasation of lymphocytes to extracellular sites. Access to integrins after they bind high-affinity ligands, however, may be limited, or it may be too late to disrupt tight binding, and a nonselective antagonist might overcome this problem.

State-selective antagonists of αβ₁, however, may have an improved embryonic safety profile. Microinjection of a nonselective αβ₁ blocking antibody or continuous exposure of rat whole embryo cultures to nonselective antagonists of αβ₁ can induce defects in chorio-allantoic fusion. Although a selective antagonist of αβ₁ was evaluated in this study, the lack of potency of this compound in 90% serum made it difficult to assess the role of selective antagonism on development (Spence et al., 2002). Furthermore, although gene knockout of the β₃ subunit or VCAM-1 have been shown to result in embryo-lethality, this is not the case for gene knockouts of β₂ (Butcher, 1999). Thus, the use of small molecule ligands to measure binding parameters of αβ₁ and αβ₂ in different states of activation will advance our understanding of how to develop potent, efficacious, and safe antagonists of αβ₁ and αβ₂.

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