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Cellular Solid-Phase Binding Assay and Mass Spectrometry for Screening of α4β7 Integrin Antagonists

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Abstract—A qualitative cellular solid-phase binding assay for screening α4β7 integrin antagonists attached via photolinker to TentaGel® Macrobeads has been developed. An activation of the integrins with Mn^{2+} was necessary to achieve binding to the bead bound antagonists. The identification of the resin bound compounds was done by mass spectrometry.

Integrins α4β1- (VLA-4) and α4β7- (LPAM-1) are heterodimeric cell-surface receptors expressed on most leukocytes. By mediating cell–cell and cell–matrix interactions, they play an important role in the physiologic and pathologic response of the immune system.[1] Mucosal addressin cell adhesion molecule 1 (MAdCAM-1) is the natural ligand for α4β7-integrin. It is predominately expressed on high endothelial venules (HEV) in Peyer's patches (PP), on blood vessels within the lamina propria of the gut,[2] and on sinus-lining cells in the spleen.[3] The MAdCAM-1/α4β7-integrin interaction is crucial for lymphocyte homing to the gut.[4] α4β1-Integrins bind, even after high activation, only weakly to MAdCAM-1.[5] In mouse models of colitis and in human inflammatory bowel disease (IBD), MAdCAM-1 is over expressed.[6] Anti-α4β7-mAbs have been shown to ameliorate or prevent IBD.[7] After structural elucidation of α4β7-integrin antagonists,[8] we are currently developing small molecules for inhibiting the MAdCAM-1/α4β7-integrin interaction. For screening large libraries an efficient biological testing is necessary. Since 1991, when reported for the first time by Lam et al.,[9,10] solid-phase binding assays have been performed most commonly with soluble receptors,[11–13] but rather seldom with whole cells.[14,15] Herein, we describe for the first time a cellular solid-phase binding assay for screening α4β7-integrin antagonists.

For establishing a cellular solid-phase test system for α4β7-integrins (Scheme 1), recently reported antagonists,[16] which have already shown their biological activity in a normal cell adhesion assay, were assembled on TentaGel® Macrobeads (loading ~ 0.25 mmol/g). The compounds were anchored with a photolinker to the resin.[17] This linker was stable to repeated basic and acid treatment, but allowed cleavage with UV-light at 365 nm. The compounds were synthesized using the *N*-Fmoc/*tert*-butyl ester strategy and HATU/HOAt/Collidine in DMF as coupling reagents.[18] Using TentaGel® Macrobeads required prolonged coupling times (1.5 h) and more washing steps. TentaGel® Macrobeads had to be treated very gently to avoid breaking. The side-chain deprotection was effected with a mixture of TFA/water/triisoproylsilane (95/2.5/2.5 v/v) for 1 h. To remove all organic and acidic solvents, the resin was washed with DCM (3×10 min), 20% DIEA in DCM (3×5 min), DCM (3×5 min) and diethylether (3×5 min). Prior to use, the resin was dried under high vacuum. As Table 1 shows, compound **1** represents a known antagonist attached to TentaGel® Macrobeads with a photolinker.[16] Compound **2** contains an additional amino acid to reduce the influence of the photolinker. Compound **3** represents a random sequence. The identity of all peptides was confirmed by HPLC and HPLC-ESI-MS.

The results of the biological testing are shown in Figure 1. 38C13β7 B-cell lymphoma cells ($\alpha4\beta7^+$, $\alpha4\beta1^-$) adhered in a monocellular layer to TentaGel® Macrobeads

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carrying compounds **1** and **2**. The photolinker connected directly to the C-terminus of the antagonist, had no negative influence on the testing. There was nearly no cell adhesion to the random sequence **3**. If mixing compound **1** and **3** (ratio 1:3) it was very easy to distinguish between beads carrying cells and empty beads. Without activation of the α4β7-integrins with 1 mM Mn^{2+} only very few cells adhered to compound **1**. The activation with 1 mM Mn^{2+} was also sufficient for Jurkat cells (α4β7$^-$, α4β1$^+$) to adhere to compounds **1** and **2**, but not to the random sequence **3** (Fig. 2).

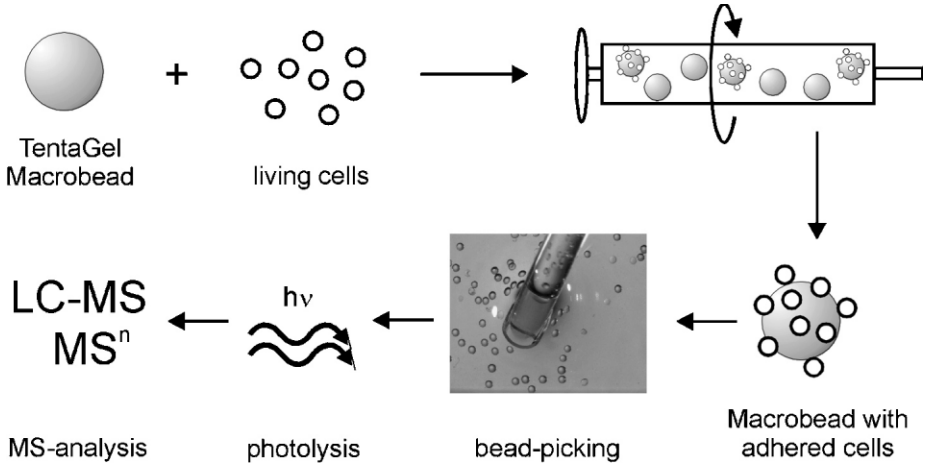
After scanning with the microscope, the beads were picked with a pipette, transferred into a syringe, and washed several times with a solution of 1 mM EDTA and adhesion buffer. The compounds were released from the beads in CH_3CN/water (3:1) by photolysis for 3 h. ESI-MS allowed to determine the sequence of the tested compounds (Fig. 3).

Cellular Solid-Phase Binding Assay

To avoid unspecific binding, the TentaGel® Macrobeads had to be blocked for 1 h at 37 °C with adhesion

Table 1. Synthesized compounds

No.	Sequence
1	Isochinolin-3-carbonyl-Leu-Asp-Thr-Photolinker-Macrobead
2	Isochinolin-3-carbonyl-Leu-Asp-Thr-Gly-Photolinker-Macrobead
3	Isochinolin-3-carbonyl-Leu-Thr-Asp-Photolinker-Macrobead



Scheme 1. Schematic procedure for a cellular solid-phase binding assay with mass spectrometric analysis of the compounds.

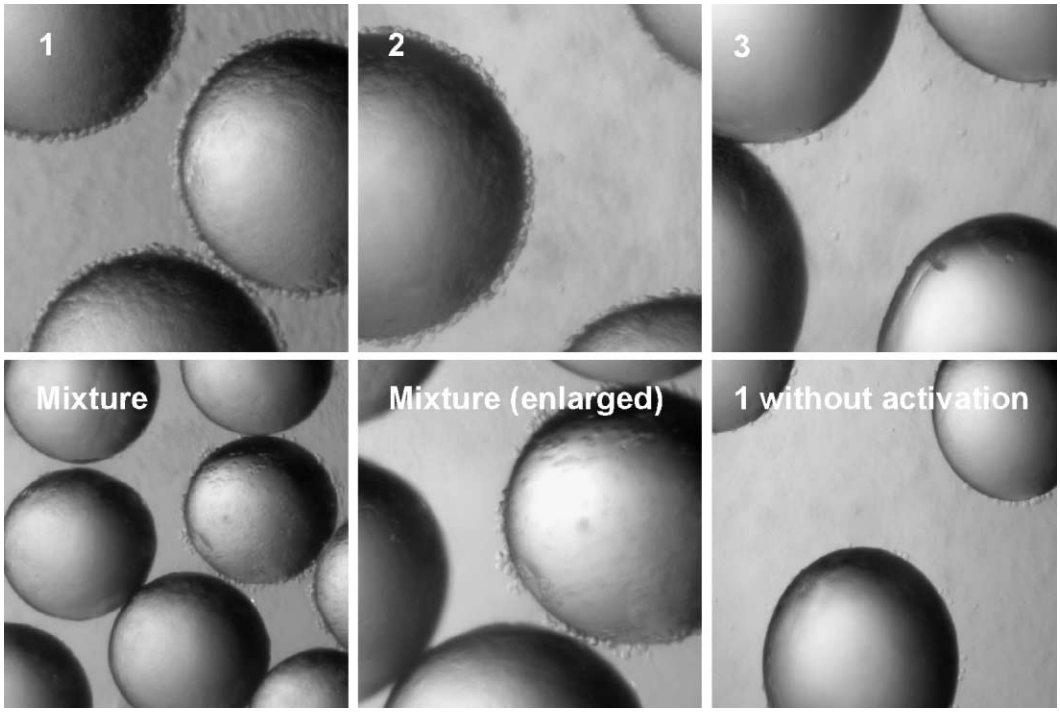


Figure 1. Compounds **1**, **2**, **3**, and a mixture of **1**+**3** (ratio 1:3) were incubated with activated 38C13β7 B-cell lymphoma cells (α4β7$^+$, α4β1$^-$). 38C13β7 B-cell lymphoma cells without activation nearly do not adhere to compound **1**.

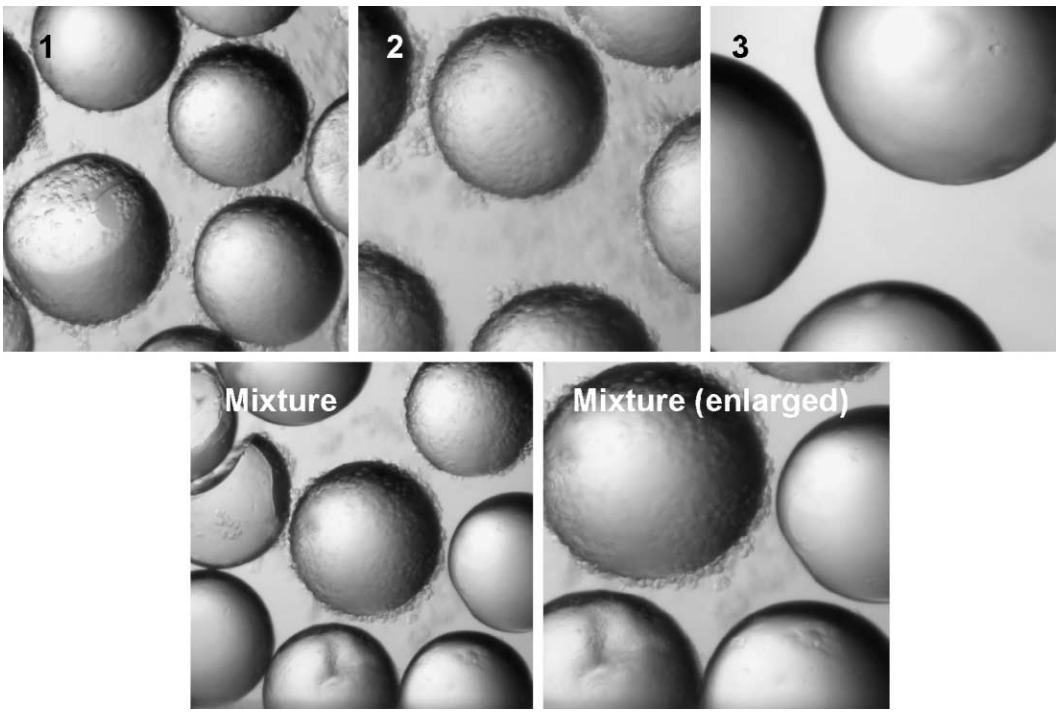


Figure 2. Compounds **1**, **2**, **3**, and a mixture of **1** + **3** (ratio 1:3) were incubated with activated Jurkat cells ($\alpha4\beta7^-$, $\alpha4\beta1^+$).

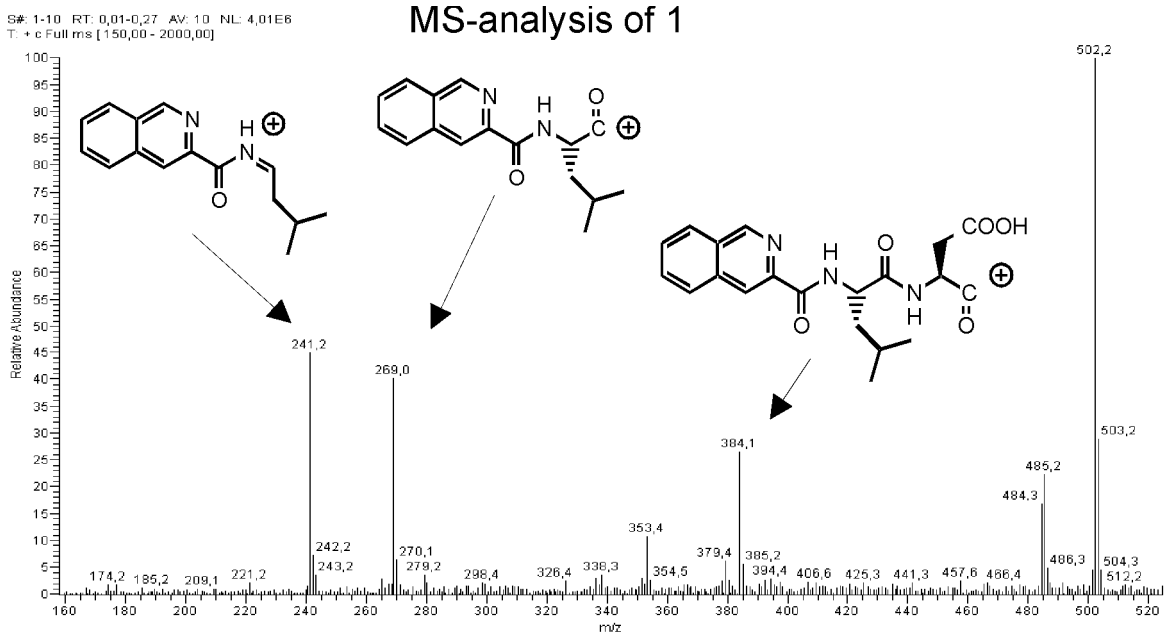


Figure 3. MS analysis of compound **1**. Sequence can be identified by ESI-MS.

buffer (Click's RPMI medium, 1% BSA, 1.0 mM $MgCl_2$ and 1.0 mM $CaCl_2$). After using different conditions, it turned out, that we got the best results by incubating 10–15 mg resin with 1.5 mL cell suspension (38C13β7-cells: 5×10^6 cells/mL or Jurkat-cells: 1×10^6 cells/mL) in TBS-puffer (14 mM Tris, 137 mM NaCl, 2.7 mM KCl, 2.0 mM Glucose and 1 mM $MnCl_2$). The incubation was done for 1.5 h at 37 °C in a 2 mL syringe with a commercially available frit. The pores of the frit were big enough, so that the cells could pass through. It was beneficial to rotate the syringes horizontally (8 rpm), so that the beads rolled over the cells. After removing the plunger, the cell-Macrobead suspension was poured into a Petri dish and analyzed under a microscope. Scanning through the beads with a microscope allowed to distinguish between loose cells and adhered cells.

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