# Selectivity of Molecular Recognition Displayed by Monoclonal Antibodies as Compared to Receptors – a New Approach to Screen Combinatorial Libraries

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Dedicated to Professor *Duilio Arigoni* on the occasion of his 75th birthday

Photolabeled monoclonal antibodies generated against a potent antagonist of the glycoprotein receptor GPIIb-IIIa have been used to demonstrate that antibodies can be employed to replace receptors for on-bead screening of combinatorial libraries.

**Introduction.** – High-throughput screening of combinatorial libraries requires a fast and reliable binding assay to a purified and stable receptor [1]. In practice, this aim is often difficult to accomplish due to technical problems regarding the solubility of the receptor, its interactions with compounds linked to a polymer, and the selectivity of the binding assay over a broad range of binding constants.

In recent years, considerable progress has been achieved creating artificial receptors by 'imprinting' synthetic polymers [2–4]. Another alternative would be to use polymers 'imprinted by nature' such as monoclonal antibodies generated against one of the possible target compounds as a replacement for the natural receptor. In this communication, we wish to present our first results concerning the proof of concept.

**Results and Discussion.** – To investigate the suitability of antibodies for selective recognition, we chosed lamifiban (**1**), a known antagonist [5] of the GPIIb-IIIa receptor belonging to the integrins [6], which are cell-surface receptors participating in the aggregation of thrombocytes [7–9]. Conveniently, a monoclonal antibody (mAB-LMF-2) generated against **1** was available as well as several analogues **2**–**10** [5][10] (*Fig. 1*), for which the binding constants to the receptor are known to cover a range of three orders of magnitude. Antagonists **1**–**10** have one structural feature in common, *i.e.*, the COOH group is used to link the compounds to beads. The PEGA resin [11] was chosen as a suitable bead material because the polymer allows for diffusion of large biomolecules $< 70$ kD, and beads swollen in buffer have a size of 300–500 μm. Coupling of antagonists to PEGA resin [12] was performed according to *Scheme 1*, yielding a final concentration of *ca.* 200 μmol/g resin.

The monoclonal antibody mAB-LMF-2, generated against **1**, was derivatized at its lysine $NH_2$ groups with the commercially available, *N*-succinimide-activated fluores-

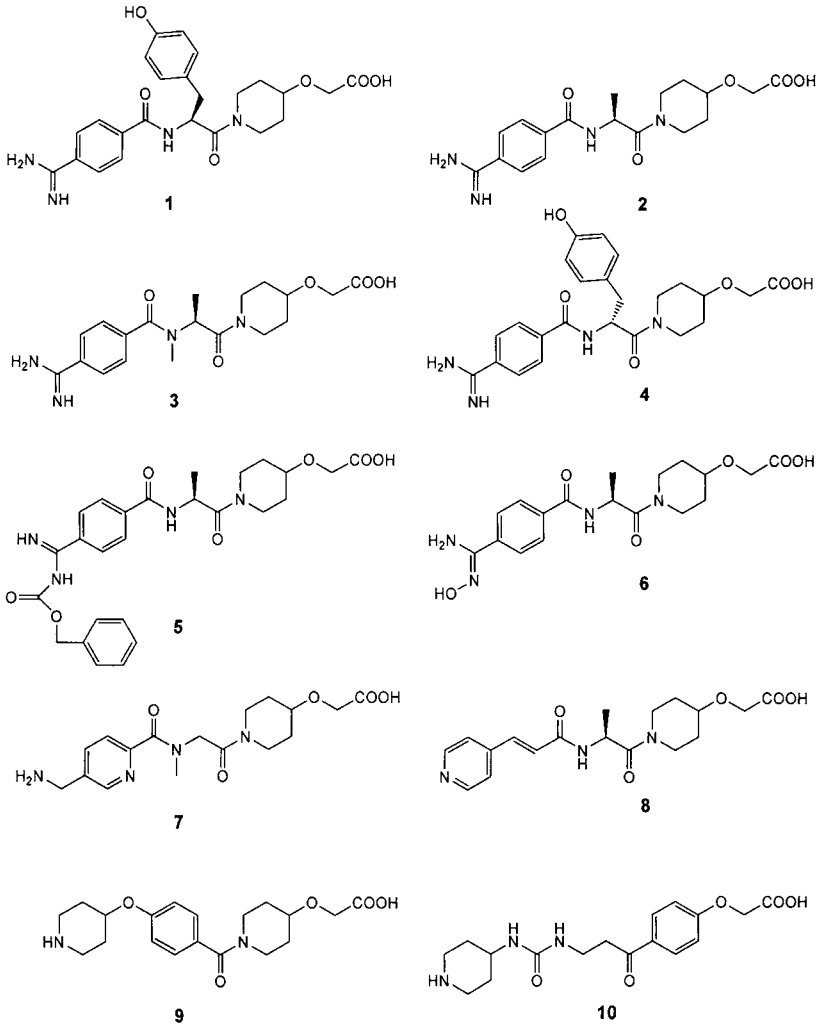
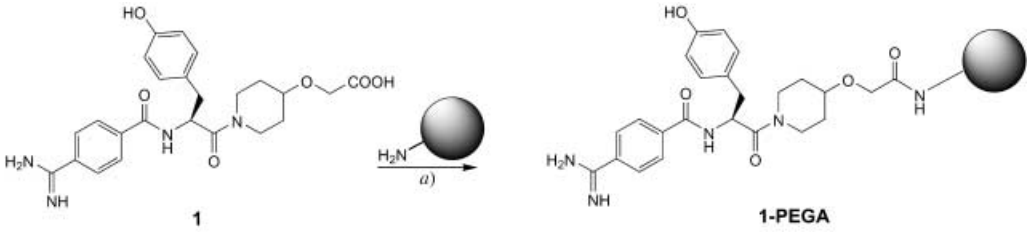



Fig. 1. *GPIIb-IIIa Receptor antagonists*

cence label *Alexa 546* (**11**) [13] (*Scheme 2*; $\lambda_{abs}$ 558 nm, $\lambda_{em}$ 573 nm) yielding an epitopic density of 2.5 on mAB-LMF-2-**11**.

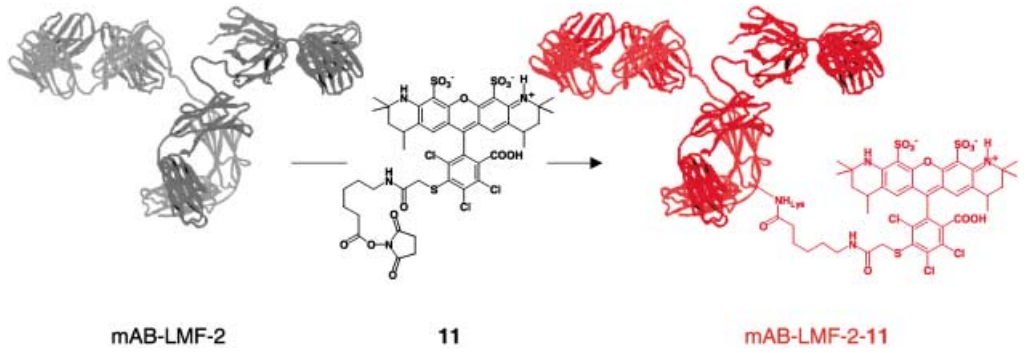
To determine the affinity of mAB-LMF-2-**11** for on-bead compounds, antibodies (100 µl of a 0.1µM mAB solution) were incubated in phosphate buffer (pH 7.2) with *ca.* 100 beads for 24 h. The 100 beads carried *ca.* 40 nmol of antagonist and the antibody solution *ca.* 10 nmol of mAB-LMF-2-**11**. This ratio and the molar concentration of the antibody are suitable to distinguish $K_D$ values in the µM to nM region [14]. After washing, the beads were embedded in glycerol to prevent diffusion and desiccation, *i.e.*, to slow down dissociation of antibodies from compounds on beads and to maintain a uniform bead structure for fluorescence analysis. The intensity of bead fluorescence

Scheme 1. *Coupling of* **1** *to the PEGA Resin*




*a*) NMP (1-methylpyrrolidin-2-one), HATU (*O*-(1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate), $^{i}$PrEtN, 2 h, 25°.

Scheme 2. *Photolabelling of the Monoclonal Antibody mAB-LMF-2 Generated against* **1**




was measured with a fluorescence microscope coupled to a CCD (charge-coupled device) camera containing respective software for statistical evaluation. The method is described in *Fig. 2:* from photos of several bead samples (*ca.* 20 beads), 5 beads were selected, and each bead was dissected into four zones, $n_1 - n_4$ (pixel lines), to obtain the average fluorescence of each bead. From this, the median value of the beads was calculated and the background intensity substracted.

By this procedure, one gains relative intensities corresponding to relative affinities of compounds **1**–**10** for the binding site of the monoclonal antibody generated against **1**. A comparison of these fluorescence intensities with $IC_{50}$ values of compounds **1**–**10** of the GPIIb-IIIa receptor is shown in *Fig. 3*.

The data in *Fig. 3* demonstrate that the antibody mAB-LMF-2-**11** recognizes receptor antagonists selectively and with a tendency similar to the GPIIb-IIIa receptor.

Absolute values of affinities of compounds **1**–**10** to the monoclonal antibody mAB-LMF-2 were obtained by binding assays performed with *Biacore* (surface plasmone resonance) [15]. The mAB-LMF-2 was immobilized on a CM5 sensor chip and treated with solutions of different hapten concentrations (**1**–**10**); time-resolved epitopic-density determination gave $k_{as}$, and subsequent washing of the loaded antibodies yielded $k_{dis}$. From these values, $K_A$ and $K_D$ of compounds to monoclonal antibody were calculated (see the *Table*).

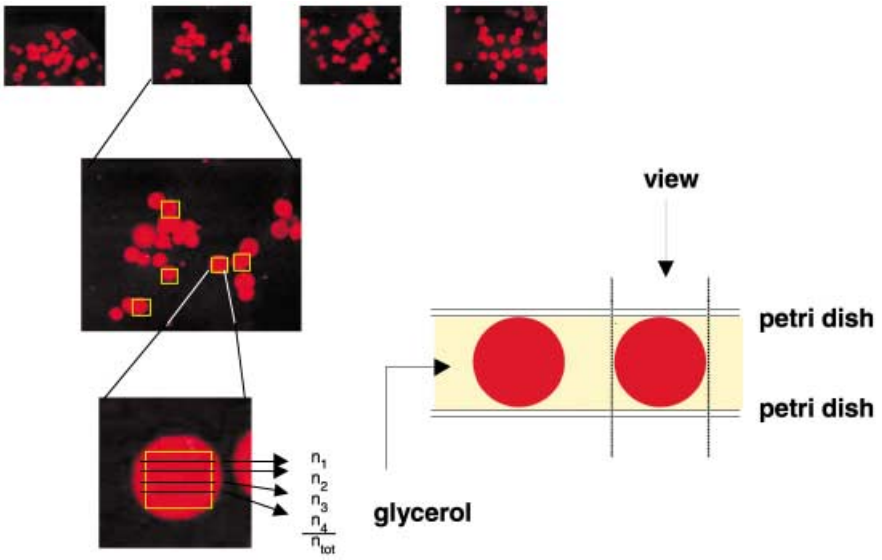



Fig. 2. *Determination of fluorescence intensities of PEGA beads carrying* **1** *after incubation with mAB-LMF-2-***11**

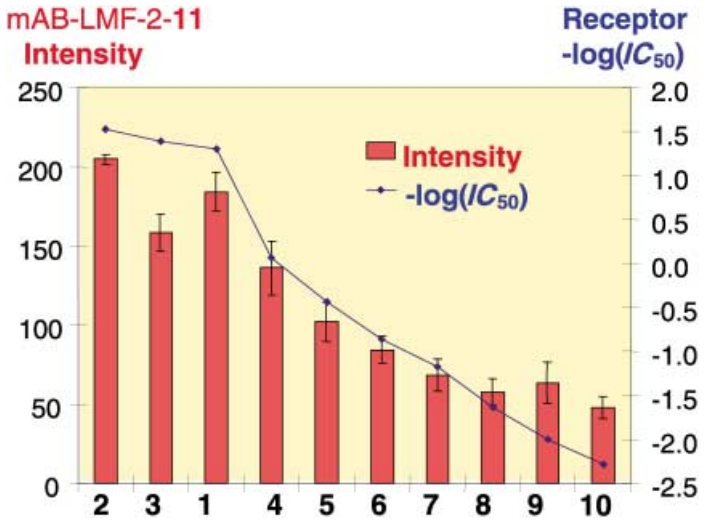



Fig. 3. *Fluorescence intensity* (red bars) *of several beads* (median intensity) *loaded with* **1**–**10** *compared to* $-\log IC_{50}$ *of the same compounds to the natural GPIIb-IIIa receptor* (blue dots/line)

A comparison of the binding constants $K_A$ of compounds **1**–**10** for the antibody mAB-LMF-2 with $IC_{50}$ of the same compounds and the GPIIb-IIIa receptor revealed a good correlation (*Fig. 4*) of molecular recognition of antibody and receptor.

Table. $IC_{50}$ *Values of* **1**–**10** *and the GPIIb-IIIa Receptor and Binding Constants of the Same Compounds for the Antibody mAB-LMF-2*

| GPIIb-IIIa-Receptor antagonist | $IC_{50}$ [μM] (ADP) | $K_A$ [$M^{-1}$] | $K_D$ [M] |
|---|---|---|---|
| **1** | 0.05 | $3.7 \cdot 10^8$ | $2.7 \cdot 10^{-9}$ |
| **2** | 0.03 | $5.6 \cdot 10^8$ | $1.8 \cdot 10^{-9}$ |
| **3** | 0.041 | $1.8 \cdot 10^6$ | $5.6 \cdot 10^{-7}$ |
| **4** | 0.85 | $2.6 \cdot 10^7$ | $3.8 \cdot 10^{-8}$ |
| **5** | 2.73 | $2.2 \cdot 10^6$ | $4.5 \cdot 10^{-9}$ |
| **6** | 7.3 | $3.5 \cdot 10^5$ | $2.8 \cdot 10^{-6}$ |
| **7** | 15.1 | $4.0 \cdot 10^4$ | $2.5 \cdot 10^{-5}$ |
| **8** | 42.8 | $2.9 \cdot 10^5$ | $3.4 \cdot 10^{-6}$ |
| **9** | > 100 | $4.0 \cdot 10^3$ | $2.5 \cdot 10^{-4}$ |
| **10** | > 194 | no specific binding | |

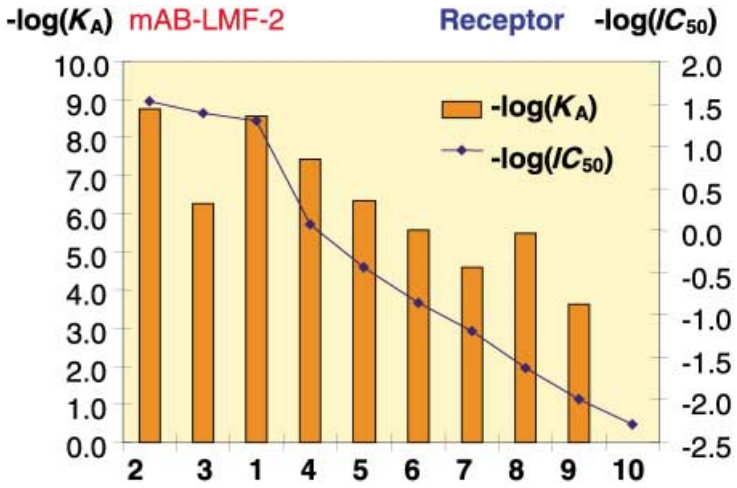



Fig. 4. $-log K_A$ (Association constant of antagonist to mAB-LMF-2) *values determined by means of* Biacore (orange bars) *and* $-log\ IC_{50}$ *values of* **1**–**10** *to the natural GPIIb-IIIa receptor* (blue dots/line)

These experiments qualitatively confirm the results obtained with the fluorescence-labelled antibody and on-bead compounds. Accordingly, two independent techniques support the idea that antibodies can replace receptors to identify compounds that have rather small structural differences.

This result is quite remarkable considering the different interactions between, *e.g.*, **1** and the GPIIb-IIIa receptor and the antibody mAB-LMF-2, respectively. Results obtained with other lamifiban analogues (not shown) suggested that the distance between the amidine unit and the COOH group is significant for binding to the receptor. For the antibody, the situation seems to be quite different. The similar affinities of **1** for mAB-LMF-2 and of **1**-PEGA (terminal carbonyl group linked to resin) for mAB-LMF-2-**11** suggests that the dominant recognition site of **1** for the antibody is the protonated amidine subunit. Nevertheless, there is significant selective recognition of the antibody to changes at the central amide moiety of **1** resembling molecular recognition displayed by the GPIIb-IIIa receptor.

In conclusion, our results indicate that antibodies can be used instead of receptors to screen split-and-mix libraries for new lead compounds.

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## Experimental Part

*General.* Receptor antagonists were coupled to PEGA resin (PL-PEGA, 0.2 mol-equiv./g, *Polymer Labs.*); solvents and reagents (*puriss.*) were commercially available and used without further purification. GPIIb-IIIa receptor antagonists **1**–**10** and the monoclonal antibody mAB LMF-2 were supplied by *F. Hoffmann-La Roche Ltd.* Fluorescence labelling of the antibody mAB-LMF-2 was pursued with the *Alexa-Fluor*™*-546* protein-labeling kit (*A-10237* from *Molecular Probes*). To determine the fluorescence intensity of the PEGA beads, the stereomicroscope *Leica MZ* 12 was employed which was equipped with a *GFP-Plus* fluorescence lamp and a CCD camera *Leica 2*. To quantify fluorescence intensities, the following software was used: Image Access, version 2.05 (*Imagic Bildverarbeitung AG*, Glattbrugg), Leica QWin, standard version 2.1 (*Leica Imaging Systems*). The association constants of compounds to antibody were determined with *Biacore® 2000* und *Biacore® HS*.

*Coupling of Compounds* **1**–**10** *to PEGA-Resin Beads.* The compound (3 equiv.) was added to the PEGA resin (*ca.* 0.2 mmol ($-NH_2$)/g resin) together with HATU (*O*-(1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; 3 equiv.), and $^{i}Pr_2EtN$ (10 equiv.). By addition of NMP (1-methylpyrrolidin-2-one), the total volume was adjusted to *ca.* 1.5 times the volume of the resin. The mixture was stirred for 2 h at r.t. and subsequently washed with $^{i}$PrOH and MeOH.

*Binding of Antibody mAB-LMF-2-***11** *to On-Bead Compounds and Determination of Fluorescence Intensities.* A sample of resin (250 µl; epitopic density of antagonists *ca.* 2.5) was suspended in a 2-ml *Eppendorf* tube and washed with phosphate buffer (pH 7.2). Subsequently *ca.* 100 beads were transferred to a microtiter plate, 0.1 µM mAB-LMF-2-**11** soln. (100 µl) was added, and incubation was performed for 24 h at r.t. The mAB soln. was then removed, and the beads were briefly washed with phosphate buffer (1 × 100 µl) and finally covered with glycerol (200 µl) and embedded between two petri dishes, see *Fig. 2*.

$IC_{50}$ *Values.* Inhibition of ADP-induced platelet aggregation was performed according to [5].

$K_D$ *Values.* The monoclonal antibody mAB-LMF-2 was immobilized on a CM5 sensor chip (research grade) in cell 2/3; cell 4 of the *Biacore* contained the immobilized anti-amyloid-antibody BAP1 as reference to compensate for unspecific binding and change refraction. The immobilization was done according to a *Biacore* protocol by using EDC (*N*-[3-(dimethylamino)propyl]-*N*′-ethylcarbodiimide hydrochloride) and NHS (*N*-hydroxysuccinimide) for surface activation. Excess of activated surface carboxy groups were quenched with 1.0M 2-aminoethanol (pH 8.5).

The mAB-LMF-2-modified surface was then contacted with various concentrations of each antagonist **1**–**10** and the change of epitopic density measured as a function of time yielding $k_{as}$. The dissociation constant was obtained by time-controlled removal of antagonists through washing with phosphate buffer.

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