

# Lead selection of biologics with low clearance due to receptor-mediated endocytosis

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# Lead selection of biologics with low clearance due to receptor-mediated endocytosis

Researchers at Swedish Orphan Biovitrum used Biacore<sup>™</sup> T200 and IN Cell Analyzer 2000 to identify new potential therapeutics with the preferred structural requisites to prevent clearance through receptor-mediated endocytosis. Binding interactions between lead protein constructs and low-density lipoprotein receptor-related protein-1 (LRP-1) were identified and characterized using Biacore T200. Good correlation of the data show the characterization of receptor-binding and glycan profiling are effective tools for the selection and development of new leads with low clearance. Lack of lysosomal uptake was then verified in cell assays using IN Cell Analyzer 2000.

### Introduction

Secreted proteins are constantly replenished by cellular uptake and recirculation to the cell surface. Therapeutic proteins given by systemic administration are similarly metabolized and their pharmacokinetic profiles are therefore reflected by their cellular uptake and in the case of smaller proteins, their filtration through the kidneys. Uptake and cellular disposal in lysosomes can be active and proteinspecific through receptor-mediated endocytosis or passive through fluid phase pinocytosis.

We have studied the structural determinants for receptormediated uptake of lead proteins with different characteristics in terms of sites recognized by scavenging receptors. Our hypothesis is that a reduction in the receptor-specific interactions studied will translate into an increased plasma residence time and higher efficacy.

Previous in-house studies have shown that our protein lead is cleared through mechanisms dependent on scavenger receptors of the low-density lipoprotein receptor family. Low-density lipoprotein receptor-related protein-1 (LRP-1) (Fig 1A) is one of the most prominent in the family with over 50 ligands described (1, 2) and can act as a co-receptor to accelerate uptake mediated by other receptors.



**Fig 1.** (A) Schematic representation of the LRP-1 receptor with clusters I–IV highlighted. (B) Principal of cell-mediated clearance.

The objective of this study was to select lead molecules with preferred structural requisites to prevent clearance through receptor-mediated endocytosis and subsequent transport to the lysosome (Fig 1B). We have used binding profiles *in vitro*, using Biacore T200, as a deselection tool to find new leads with low clearance and we have verified lack of uptake and transport to the lysosome compartment in cell assays using IN Cell Analyzer 2000 (Fig 2).



**Fig 2.** Project work flow illustrating selection of protein constructs with low receptor binding and high potency, followed by evaluation of cultivation conditions resulting in leads with the desired characteristics.

## Method

Experiments were performed using a Biacore T200 system (GE Healthcare) and an IN Cell Analyzer 2000 (GE Healthcare).

#### Biacore T200

The Biacore T200 analysis temperature was 25°C and sample compartment temperature was 15°C.

Receptors were amine-coupled in the active flow cell of a Series S Sensor Chip CM5 (GE Healthcare) according to the manufacturer's instructions. LRP-1 clusters II (68.1 kDa), III (72.8 kDa) and IV (MW 76.6 kDa) (R&D Systems) and Lectinlike receptor (Swedish Orphan Biovitrum) were diluted in 10 mM sodium acetate, pH 4.5-5.0, obtaining immobilization levels in the range 4000 to 8000 RU for binding studies and approximately 600 RU for kinetics.

Following each experiment both active and reference flow cells were regenerated using a 30 s injection of 300 mM NaCl and 10 mM sodium acetate, pH 5.0.

Data were double referenced, first by subtraction of reference flow cell and then subtraction of blank cycles.

#### IN Cell Analyzer 2000

#### Sample preparation

Fibroblasts (primary cells from patients with the disease of interest) were seeded in complete DMEM growth medium (15% FBS) at a density of 6000 cells/well in Greiner Bio-One 96-well cell culture microplates (Bionordika). Cells were treated with Alexa Fluor™ labeled proteins of interest at the time for seeding. Untreated cells and an irrelevant labeled protein served as controls. The plates were then incubated for three days at 37°C and 5% CO<sub>2</sub>.

#### Image acquisition

The living cells were stained for detection of nuclei (Hoechst 33342), lysosomes (LysoTracker™ Red, Life Technologies), and in some cases cytoplasm (CellMask™, Life Technologies) immediately before image acquisition. Cells were then imaged using a 20× objective and twelve fields of view were acquired from each well using the DAPI\_DAPI filters for Hoechst 33342 (nuclei) and the FITC\_FITC (labeled protein) and filters for Texas Red™ (lysosomes). Three or four channels were used and analyzed at the same time. Conditions for the different channels were chosen while looking at the living cells when placed in the instrument (+CO<sub>2</sub>).

#### Microscopy

Binning  $1 \times 1$ , Quad 2

- 1. DAPI (for Hoechst) Image: 2D, Exposure: 0.100, Offset: 3.00
- 2. Texas Red (for Lysotracker Red) Image: 2D, Exposure: 0.350, Offset: 5.00
- 3. FITC (for Alexa Fluor 488) Image: 2D, Exposure: 0.100, Offset: 5.00

#### Image analysis

The high-content screening platform IN Cell Analyzer 2000 and the Developer Module were used to detect and analyze co-localization in lysosomes after treatment with the various proteins. In the protocol, nuclei were segmented using the Hoechst 33342 signal and a collar was applied to define the lysosomal and protein sampling area.

#### Results (Biacore T200) Identifying interactions with the LRP-1 receptor

In an attempt to design novel protein lead candidates exhibiting reduced interaction with the LRP-1 receptor, we identified and characterized the interaction using immobilized domains of LRP-1 receptor (Fig 3).

Direct interaction with LRP-1 cluster IV could be confirmed for both the positive control protein RAP (R&D Systems) and the protein of interest. Binding to cluster III was observed with the positive control whereas our protein candidate showed no binding. Our positive control did not bind cluster II in this experiment indicating a lack of active receptor fragment in this preparation.



**Fig 3.** Sensorgrams showing binding of receptor associated protein (RAP) and a protein candidate to LRP-1 cluster II, III, and IV.

#### Site specificity

Competition experiments were performed to study whether our protein constructs bound to the same site on LRP-1, cluster IV, as RAP. Prior to this experiment increasing concentrations of RAP were injected over the LRP-1 receptor surface verifying that a RAP concentration of 0.8  $\mu$ M saturated the immobilized receptor (not shown). The studied protein was then injected over the surface at 1.6  $\mu$ M. Finally a mixture of protein and RAP, both at 1.6  $\mu$ M, was injected (Fig 4).

If the protein construct and RAP bound to different sites we would expect a result similar to the dashed curve. The sensorgram data representing the mixture of RAP and protein contains the sum of both interactions and resembles that of RAP alone. This indicates that our protein of interest shows a specific interaction with the receptor that can be displaced by RAP.



Fig 4. Sensorgrams showing binding to immobilized LRP1 cluster IV.

#### Kinetics for the LRP-1 cluster IV interaction

LRP-1 cluster IV binding was studied using single cycle kinetics experiments (3). Fifteen protein constructs were evaluated for binding to the immobilized cluster IV domain of LRP-1 and ranked according to binding characteristics. Constructs with the concentrations 50, 100, 200, 400, 800, and 1600 nM were injected over a surface with 600 RU LRP-1 immobilized (Fig 5).

Constructs with limited interaction with LRP-1 cluster IV were selected for further characterization.



**Fig 5.** Example showing evaluation of LRP-1 cluster IV binding of four different protein variants. Protein modifications #2 and #3 result in decreased binding kinetics.

#### Defining N-glycan profiles

Over-expression of glycosylated proteins can, due to restrained resources of the post translational machinery, result in production of protein with immature N-glycan structures. These structures are recognized by scavenger receptors, which results in rapid clearance of the protein.

Twenty protein batches from an initial expression feasibility study were analyzed in a receptor binding assay. Figure 6 shows the interactions of nine of these constructs.



**Fig 6.** Binding to the glycan receptor exemplified by nine protein batches with significantly different characteristics.

Glycan profiles could be evaluated by qualitative ranking of responses. A large variation in the degree of binding to the glycan-recognizing receptor was apparent and correlated well to the degree of glycan maturity as determined by LC-MS for selected protein batches.

The data show this assay could be used to define a preferred glycan profile, which was a selection criterion for further development.

#### Glycan receptor-binding assay reproducibility

The glycan receptor chip could be used on multiple assay occasions. Batch #7 was used as working standard and the response for this batch was concluded to be reproducible between assays (Fig 7).

The assay showed excellent reproducibility with low inter-day variability of the working standard.



Fig 7. Overlay plot showing sensorgrams of the working standard (batch #7) run on days 1 to 4.

#### **Binding specificity**

To check binding to the immobilized lectin-like receptor is specific, the same concentration of protein construct #7 was mixed with increasing concentration of a particular end-glycan motif and injected over the receptor. Specificity of the interaction could be confirmed by displacement of protein batch #7 by the glycan in a concentration-dependent manner (Fig 8).



**Fig 8.** Sensorgrams showing decrease in protein batch #7 binding to immobilized glycan receptor as the concentration of a soluble end-glycan displaces protein binding.

### Results (IN Cell Analyzer 2000) Endocytosis studies

The IN Cell Analyzer 2000 was used to study uptake and lysosomal disposal of lead molecules and to correlate results with receptor binding.

Selected protein batches were functionally characterized through their intra-cellular localization in primary fibroblasts. As an example, one of the Alexa Fluor™ 488-labeled protein constructs was found throughout the endosomal network, as evidenced by a punctuated cytosolic fluorescence pattern, and co-localized with lysosomal staining (Fig 9).



**Fig 9.** Primary fibroblasts imaged with IN Cell Analyzer 2000. Four colors were detected: nuclei (blue), lysosomes (yellow), protein batch #3 (turquoise) cytoplasm (deep red).

#### Decrease in endocytosis of lead proteins

Two protein variants were selected based on their interactions with LRP-1 and the glycan recognition receptor. Specific intracellular protein signal and co-localization of signal with lysosomes were analyzed (Fig 10). Less co-localization with lysosomes is seen for batch #2 as compared with batch #7, indicating a correlation of these results with receptor binding studied in Biacore T200.

#### **Receptor-binding characteristics**

The degree of uptake into primary fibroblasts was found to correlate with the LRP-1 binding characteristics of several protein variants. This is illustrated using data from one high and one low LRP-1 binder lead protein (Fig 11). Also co-localization with the lysosomal compartment was lower for the protein with lower receptor binding.



**Fig 11.** Degree of uptake for protein concentration series measured as A. Total intracellular counts, and B. Normalized co-localization.

(A)



Fig 10. A. High binder, protein batch #7). B. Low binder, protein batch #2. Colors are: nuclei (blue), lysosomes (red), protein (green), lysosome/protein co-localized (yellow).

#### LRP-1 expression in primary fibroblasts

Western blot results confirmed the presence of LRP-1 on the fibroblasts used in the studies using IN Cell analyzer 2000 (Fig 12).



Fig. 12. Western blot with arrow showing the presence of LRP-1.

#### Inhibition of uptake by known LRP-1 ligands

To verify that the endocytosis seen was dependent on the LRP-1, receptor cells where incubated for 24 h in DMEM supplemented with 75 nM of protein alone or in combination with 760 nM of a known LRP-1 ligand (Fig 13).



Fig 13. Inhibition of endocytosis by LRP-1 ligands.

### Conclusion

Researchers at Swedish Orphan Biovitrum used Biacore T200 and IN Cell Analyzer 2000 throughout the protein construct phase as well as during culturing to select lead molecules with preferred structural requisites to prevent clearance through receptor-mediated endocytosis and subsequent transport to the lysosome. Results from *in vitro* receptor binding and glycan profiling were used as a deselection tool to find new leads with low clearance. Lack of uptake and transport to the lysosome compartment could be verified in cell assays using IN Cell Analyzer 2000.

#### References

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- 3. R. Karlsson, *et al.* Analyzing a kinetic titration series using affinity biosensors, *Anal. Biochem.* **349**, 136-147 (2006).

## **Ordering information**

Description	Product code
Biacore T200 Processing Unit	28975001
IN Cell Analyzer 2000, standard chip CCD camera	28953463
Series S Sensor Chip CM5, pack of 3	BR100530
Amine Coupling Kit	BR100050

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