Nanobodies and HTRF® technology to address an orphan class B GPCR: The GPR97 receptor

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INTRODUCTION The GPR97 receptor is an orphan class B GPCR with a very large multidomain N-terminal extracellular region (~200 amino acids).

The physiological roles of this receptor are not well-known, but it is thought to play a role in immune functions and neurodegenerative diseases.

Several nanobodies (single domain antibodies) were developed using a phage display selection (in collaboration with the Antibody therapeutics and immunotargeting team). A first characterization using flow cytometry experiments showed that two clones had a specific binding on GPR97 receptors and possibly recognized two different epitopes.

Cisbio Bioassays developed a SNAP-tag plasmid encoding for GPR97 receptors. The SNAP-tag is a suicide enzyme which was fused to the N-terminal position of the GPR97 receptors. Once transfected into cells, this plasmid construction lead to the expression of SNAP-GPR97 at the cell surface. SNAP-tagged receptors can be covalently labeled with the HTRF donor, terbium cryptate (Lumi4-Tb), using a specific SNAP substrate.

Several agonist and antagonist compounds had previously been selected by Sanofi in a large scale screening of an internal chemical library. In collaboration with Sanofi, these compounds were used to investigate the characterization of nanobodies in cell-based assays.

This poster shows the first proof of concept demonstrating the potential of nanobodies as new tools for GPR97 receptor investigations in combination with the HTRF technology.

VALIDATION OF SNAP-GPR97 RECEPTORS

HEK293 cells were transfected, then labeled with a specific SNAP-tag substrate coupled to the HTRF donor, terbium cryptate, developed by Cisbio Bioassays to detect cell membrane expression (Fig 1). A highly significant cell membrane expression level was detected.

Functional response of the SNAP-GPR97 receptors was tested by detecting the accumulation of second messenger cAMP, using the HTRF cAMP dynamic 2 kit in comparison with the wild type GPR-97 receptors (Fig 2). The SNAP-GPR97 remained functional after activation by an agonist compound (provided by Sanofi).

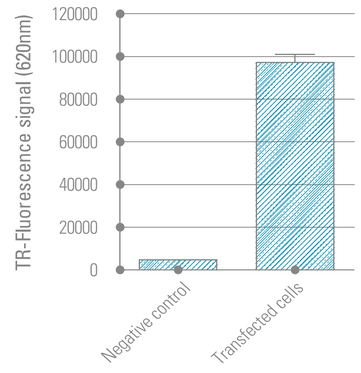

Fig 1: Validation of cell membrane expression

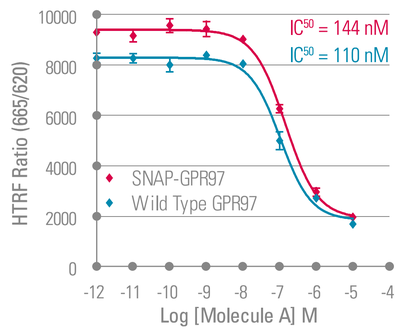

Fig 2: HTRF cAMP dynamic 2 kit assay

EVALUATION OF NANOBODIES FOR GPR97 RECEPTOR INVESTIGATIONS

Nanobodies are highly selective

Nanobody A and Nanobody B were successfully coupled to the HTRF Red acceptor. Then the binding properties of both fluorescent nanobodies were evaluated using an HTRF binding cell-based assay in an HTS compatible format (sv 384-well plate) on SNAP-GPR97 transfected HEK293 cells.

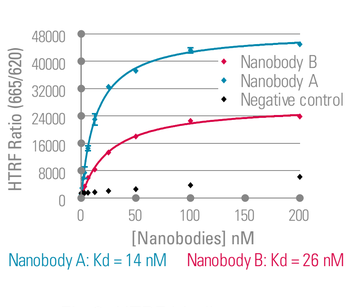

Fig 3: HTRF binding assay

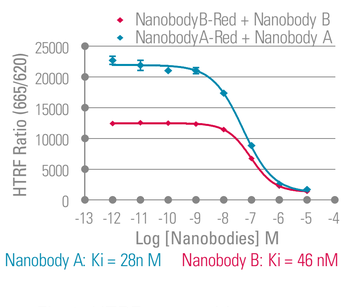

Fig 4: HTRF competition assay

Specific Kd values (Fig 3) and Ki were determined (Fig 4).The results are consistent with the Kd values measured for uncoupled nanobodies (data not shown).

GPCR CLASS A	5HTR2C	GPCR CLASS B	GIPR
	V2R		GLP1R
	CXCR4		PAC1R
	H3R		PTH1R
GPCR CLASS C	mGluR2		CALCR
RTK	EGFR		CRF1R
	HER2		GLP2R
	HER3		VPAC1R

Table 1: Test panel of membrane receptors

The selective binding of nanobodies on GPR97 receptors was tested on a large panel of membrane receptors (Table 1). No specific binding was observed, demonstrating that both nanobodies are highly selective for GPR97 receptors.

A positive allosteric modulation…

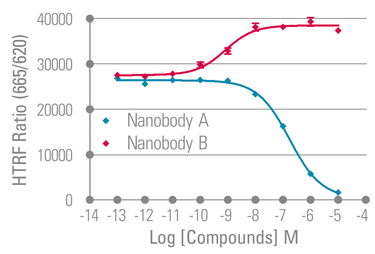

Fig 5: HTRF competition assay

To check if the nanobodies bind the same epitope an HTRF competition cell-based assay was used on SNAP-GPR97 transfected HEK293 cells. Nanobody A-Red acceptor was tested in competition with Nanobody A and Nanobody B (Fig 5). These results show that Nanobody A and Nanobody B do not recognize the same epitope. Furthermore, a positive allosteric modulation was detected on Nanobody A binding, due to the presence of Nanobody B.

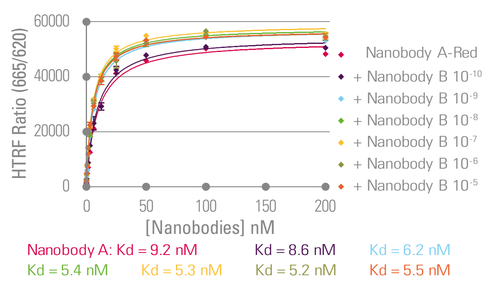

Fig 6: HTRF bindng assay to detect allosteric modulation

The Kd determination of Nanobody A-Red plus a range of Nanobody B concentrations was performed (Fig 6). The results showed a variation of the Nanobody A–Red Kd depending on the Nanobody B concentration, proving the positive allosteric modulation effect.

No pharmacological effect…

The pharmacological properties (agonist, antagonist, allosteric modulator) of the nanobodies were investigated using an agonist compound provided by Sanofi as the reference to measure second messenger accumulation by the HTRF cAMP dynamic 2 kit.

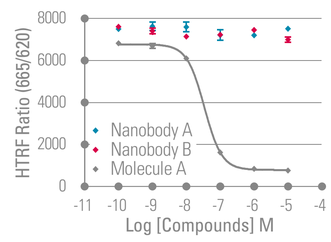

Fig 7: HTRF cAMP assay to detect agonist effect

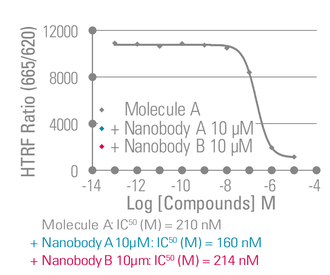

Fig 8: HTRF cAMP assay to detect antagonist or allosteric modulator effects

Investigations were performed to characterize Nanobody A and Nanobody B as agonist in comparison to the reference compound (Fig 7), and as antagonist or allosteric modulator of this reference compound (Fig 8). No pharmacological properties have been detected for either nanobodies.

EVALUATION OF NANOBODIES FOR GPR97 RECEPTOR INVESTIGATIONS

Nanobody-A Red acceptor and Nanobody-B Terbium cryptate were used as new tools to detect wild type GPR97 receptors in a homogeneous and HTS compatible format using the HTRF technology.

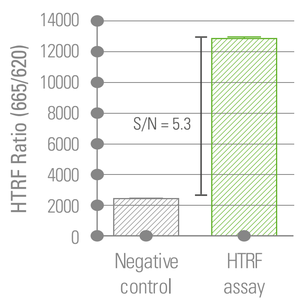

Fig 9: Expression of wild type GPR97 receptors

A cell-based HTRF assay optimization was performed to detect cell-surface expression of wild type GPR97 receptors using a GPR97-stable cell line in comparison with untransfected cells (Fig 9).

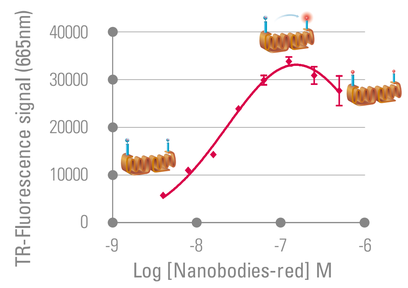

Fig 10: Homodimerization of wild-type GPR97 receptors

Finally, we performed a cell-based HTRF assay optimization to detect cell-surface homodimerization of wild-type GPR97 receptors using a GPR97-stable cell line (Fig 10).

CONCLUSION This study is the first proof of concept validating the potential of nanobodies as new detection tools, combined with the HTRF technology, to address membrane receptors.

We succeeded in developing several nanobodies recognizing different epitopes, then in coupling them to the HTRF donor and acceptor fluorophores and finally, in validating several cell-based assays.

Given these preliminary results, future experiments will mainly focus on in-vivo evaluations .

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