Project no.: 512691

Project acronym: BINDING GASTRIN

Project title: Therapeutic synthetic antibodies - binding bodies - against gastrin to treat Pancreatic Cancer

Instrument: Specific Cooperative Research Project for SMEs

Thematic Priority: Cooperative Research (CRAFT)

Publishable Final Activity Report

Period covered: from 1-10-2004 to 31-03-2007 *Date of preparation*: 03-05-2007

Start date of project: 1-10-2004 *Duration*: 24+6 months

Project coordinator name: Prof. Rob Meloen
Project coordinator organisation name: Pepscan Systems BV Version **1.0**

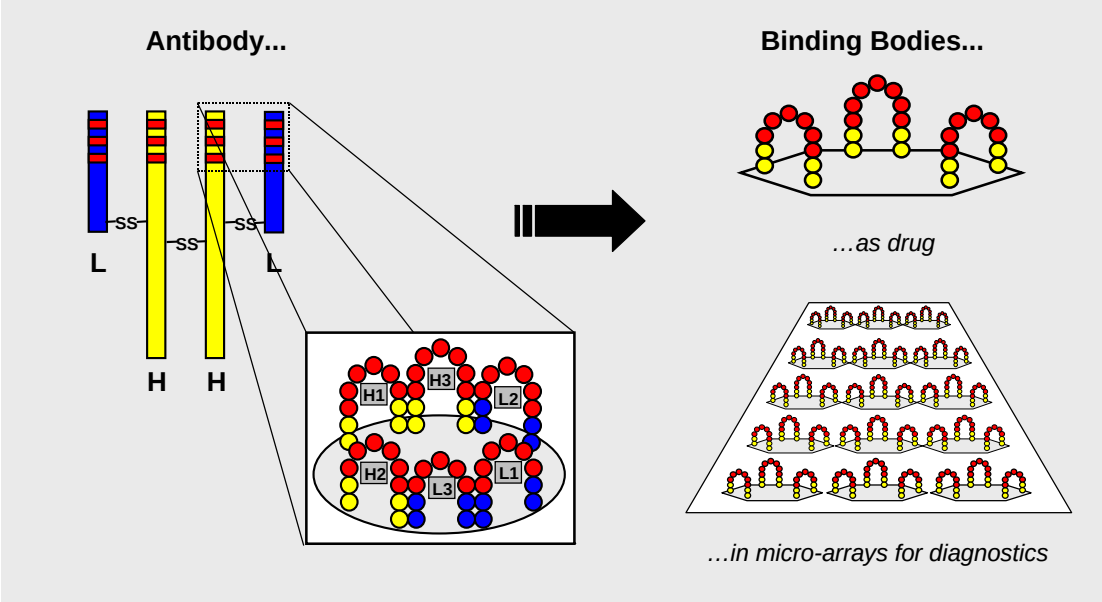
Project co-funded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
PU	Public	x
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

Project Summary

Pancreatic cancer is diagnosed 60,000 times in the USA and the EU each year. Almost the same number of people die of this disease each year. This illustrates that all existing therapies have little effect once the disease is diagnosed; survival time after diagnosis is 80 to 160 days. Gastrin drives the tumor growth.

Recently, a vaccine against gastrin is developed able to double the survival time and to improve the quality of life of pacreatic cancer patients. Unfortunately, only a limited number of individuals respond to the vaccine and produce antibodies, and even then development of antibodies is slow which is especially problematic for this fast progressing disease.

We propose to develop an alternative which will take effect immediately in all individuals treated. To this end, we propose to develop high affinity and low cost synthetic antibodies -´binding bodies´- against gastrin. Binding bodies are two or more peptides which represent CDR's (complementary determining regions, which are the hypervariable and antigen-interaction parts of the binding site of an antibody) coupled covalently to a small chemical scaffold. The concept works: ample evidence shows that peptides representing single CDR's can bind antigen. Preliminary results show that combining peptides representing at least two different CDR's give much higher binding and much more specificity. We speculate that further optimisation should yield low cost synthetic antibodies - binding bodies - with activities similar to natural antibodies.



A major and concerted effort is required to involving the integration of six different technologies:

1. recombinant monoclonal antibody technology to define CDR-sequences of gastrin-binding antibodies
2. bio-informatics technology to predict a limited and optimized set of peptides that can be used to synthesize and optimize bb's
3. peptide synthesis and peptide library technology as well as scaffolding technology for the synthesis, construction and structural optimization of bb's
4. BIACORE technology for binding affinity measurements for mAb's and bb's
5. animal model technology for *in-vitro* and *in-vivo* verification of therapeutic potential
6. peptide micro-array technology to develop gastrin-binding bb-arrays for fast selection purposes and subsequent clinical applications

The revolution of therapeutic monoclonal antibodies as a novel drug to treat a variety of malignancies is ever increasing. 18 therapeutic mAb's were approved by the FDA since their introduction in 1975 by Kohler & Milstein. Over 150 mAbs are currently in clinical study, with 63 in phase-I, 74 in phase-II, and 15 in phase-III (Nature Biotechnology 2005, 23, 1073-1078). It needs no further comment that

synthetic bb's with affinities and selectivities of those of mAb's would mean another revolution in the field.

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Contractors

Partic. Role*	Partic. Type**	Partic. no.	Participant name	Participant short name	Country
CO	SMEP	1	Pepscan Systems B.V.	**PS**	NL
CR	RTD	2	Fundacion Centro Nacional de Investigaciones Oncologicas Carlos III	**CNIO**	ES
CR	RTD	3	Universite Louis Pasteur	**ULP**	FR
CR	SMEP	4	Algonomics	**AN**	BE
CR	SMEP	5	Proteomika S.L.	**proteom**	ES
CR	RTD	6	University Medical Center Utrecht	**UMCU**	NL

*CO = Coordinator, CR = Contractor **SMEP, RTD, or OTH

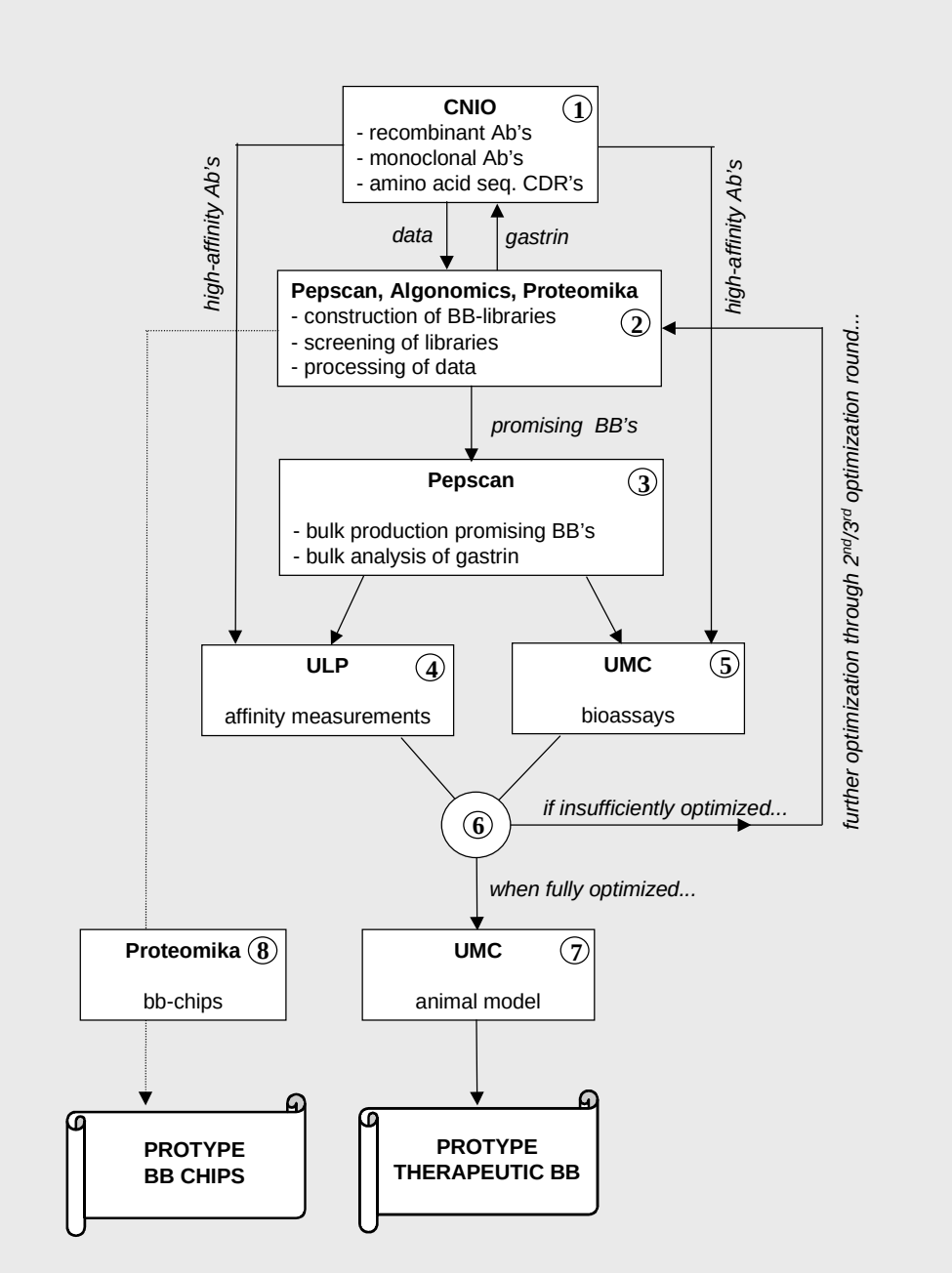
Overview of General Project Objectives

1. production of high-affinity anti-gastrin antibodies
2. production and selection of high-affinity scFv's for gastrin
3. extract sequence information for high-affinity anti-gastrin mAb's and scFv's
4. computer simulations of high-affinity mAb-gastrin and scFv-gastrin complexes
5. design of high-affinity bb's using the provided sequence information
6. development of fast and predictive procedures for the design of affinity-improved bb's based on the initial screenings
7. fast and low-cost production of bulk quantities of promising bb's for affinity measurements
8. development of relevant bioassays and an animal model to monitor progress in gastrin neutralizing activity of the bb's during the affinity maturation process.
9. construction of peptide micro-arrays of limited sets of bb's (1000-10000)

Organization of the Project (see also picture on next page)

The project starts with the generation of monoclonal antibodies (mAb's) and recombinant antibodies (scFv's) against gastrin (**CNIO**). The antibodies are identified with respect to their binding affinity (**ULP, CNIO**) and bioactivity (**UMCU, CNIO**) for gastrin. The extracted sequence information will serve as input for computer animated simulation of the antibody-gastrin complexes (**AN**). Computer modelling together with binding affinity measurements on gastrin-sequence variants (**ULP, PS**) should provide information on which amino acid residues are important/crucial for binding. This information will be used for the design and synthesis of binding bodies against gastrin. The binding bodies serve two purposes:

1. therapeutic bb's (**PS**) that bind gastrine with high affinity and selectivity in solution.
2. diagnostic bb-chips (**proteom, PS**), used to detect the presence of gastrin in biological fluids.



Work performed/results achieved:

- high-affinity mAb's and scFv's are available (**CNIO**)
- CDR-sequences of mAb's and scFv's are available (**CNIO**)
- binding affinity data for mAb's/scFv's/bb's in solution are available (**ULP**)
- simulated models for mAb-gastrin complexes are available (**AN**)
- *in vitro* and *in-vivo* bioassays to measure gastrin-neutralization are available (**UMCU**); in vitro bioassay has been extensively used for testing gastrin-neutralizing activity of scFvs/mAbs/bbs
- high-throughput screening assay for identification of anti-gastrin type-II bb's was set up and used to identify selective bb's for gastrin (**PS**)
- highly-selective bb with 100-500 microM-affinity for gastrin was synthesized in bulk (**PS**); bb is able to neutralize the bioactivity of gastrine in colo 320 WT cell-assay (**CNIO**). However, affinity is lower than foreseen at the beginning of the project.

- high-throughput platform for anti-gastrin scFvs/mAbs/bb's has been set up (**proteom**). Some of these selectively bind gastrin once printed on the slides and could be used for clinical applications.

Final conclusion of the project

It was concluded that many of the results published previously by research groups active in the field of antibody-mimicry draw a too optimistic view for solving this highly-interesting scientific problem. Published data claiming that lineair and single-loop peptides provide good mimics of antibody binding sites, with affinities only 10-100 fold lower in K_d, could not be reproduced and most likely are based upon incorrect interpretation of binding data (strong aggregation, see below). In our hands such mimics were of low-affinity and non-specific binders. Therefore, the starting point of this project was much different than expected.

Nevertheless, we succeeded in this project to provide proof-of-principle data for the development of lowMW bb's (~2.5 kDa) for the peptide-hormone gastrin based on CDR sequence information of high-affinity anti-gastrin mAb's and anti-gastrin scFv-fragments developed through this project. Previous work has always focussed on the binding of large proteins. Performing a similar result with a lowMW targets like gastrine (MW=2.5 kDa) should be regarded as a major achievement by itself. With the help of high-throughput screening and bioinformatics a new generation of binding bodies was developed that binds gastrin with 100-500 microM affinity and indeed was able to neutralize the bioactivity of gastrin in a tumor-cell bioassay.

The major hurdle to be taken when it comes to increasing the binding affinity of anti-gastrin bb's is to solve the problem of self-aggregation. Most bb's suffer from this problem and 99% of the efforts to improve affinity of bb's also increases their tendency to self-aggregate. This turned out to be the key-problem in characterizing lowMW bb's and optimizing their binding affinity. Natural antibodies hardly suffer from this problem, since evolution likely has solved this problem already long ago. The double- and triple-CDR bb's as developed in this project may provide a solid structural basis for developing high-affinity bb's. Additional studies that reveal if these bb's adopt secondary structure in solution, and if so, which structural entities are responsible for that, are desperately needed and also provide the basis for developing a scaffold with well-defined 3D-structure in solution with general applicability in the field of antibody- and in general protein-mimicry.

For therapeutic and clinical applications of anti-gastrin bb's, high-affinity binding is definitely needed. In this project, we have provided the proof-of-principle that selective recognition of gastrin with lowMW-bb's is indeed feasible. The development of high-affinity binders for gastrin is still fully realistic, However, further studies will be required to achieve this, for which the duration of this 2.5-years project was unfortunately too limited.

COORDINATOR/PARTNER 1: PEPSCAN SYSTEMS (PS)

Objectives and starting point

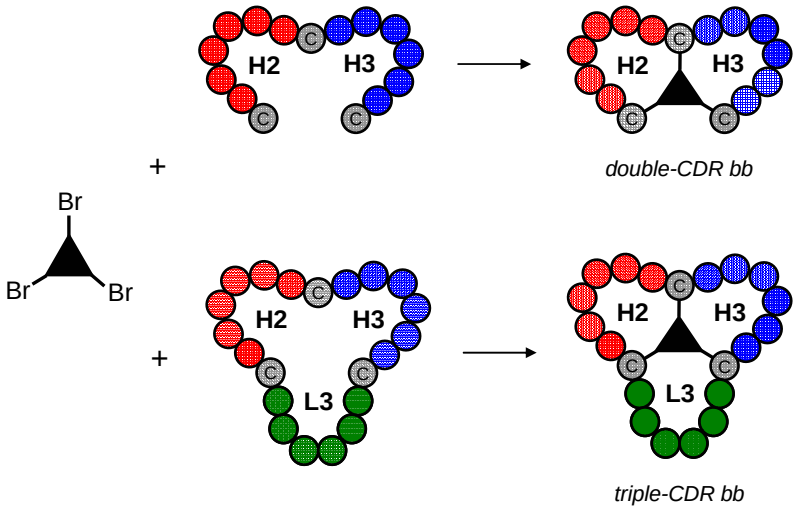
The contribution of **PS** to the « Binding Gastrin » project was, apart from coordinating the project, to provide partners with peptides for immunization (**CNIO**), SPR-studies (**ULP**), and bioassay studies (**CNIO**, **UMCU**) and bb's synthesized in bulk (**CNIO, ULP, proteom, UMCU**). Objective and starting point of the project was to translate CDR-sequence information of high-affinity anti-gastrin mAbs/scFv's produced by CNIO into small synthetic binders consisting of constrained peptide-loops on scaffolds (bb's) for high-affinity binding and selective recognition of gastrin.

Methodologies employed

- Microscale-synthesis of peptides (ng-scale) in microarrays for high-throughput-screening (HTS) purpose
- Bulk synthesis of peptides (50-500 mg scale)
- Application of CLIPS-technology for synthesis of soluble bb's (constrained double-loop peptides)
- Application of CLIPS-technology for synthesis of bb's in peptide-microarrays
- Conjugation of functionalized peptides to carrier-proteins used for immunization

Achievements made

- Successful synthesis of overlapping libraries of bb's (double-CDR-loops) for 5 anti-gastrin antibodies
- Identification of lead compounds (>5) as starting point for bulk-synthesis of bb's
- Bulk synthesis of >30 potential anti-gastrin bb's
- Identification and synthesis of 2 anti-gastrin bb's with 100-500 microM affinity for gastrin and proven selectivity over other targets (GnRH, lysozyme)
- Mapping of ~5 anti-gastrin mAb's and 2 scFv's with Ala-scan studies using Pepscan mini-libraries
- 3 manuscripts (1 submitted, 2 in preparation)



Synthesis of double- and triple-loop bb's using CLIPS-technology

PARTNER 2. FUNDACION CENTRO NACIONAL DE INVESTIGACIONES ONCOLOGICAS CARLOS III (CNIO).

Objectives and starting point

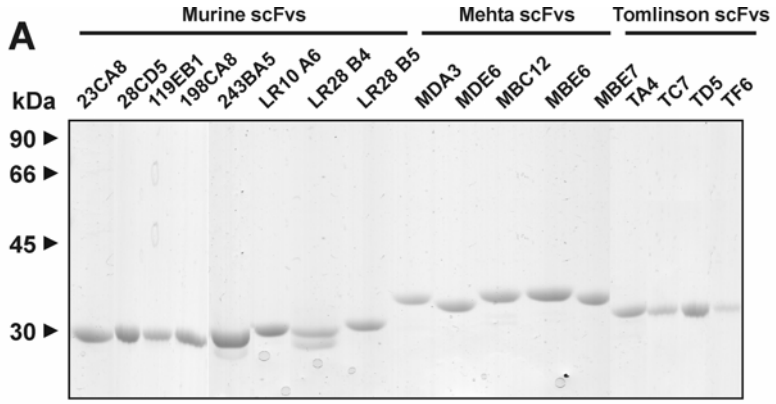
The role of **CNIO** in this project was to prepare monoclonal antibodies able to inhibit the biological activity of gastrin and use them to discover the sequences corresponding to the gastrin-binding sites (CDRs). These monoclonal antibodies are being prepared according to three different methodological approaches: i) Use of naïve human antibody libraries in phage display, ii) production of mouse monoclonal antibodies and iii) production of mouse antibody libraries from gastrin-immunised mice. Affinity maturation processes were carried out in order to produce highest affinity variants of the selected mAb's/scFv's.

Methodologies employed

- Phage display antibody libraries (Human & Mouse) production.
- Phage display selection methods
- Hybrydoma technology
- Antibody variable region cloning and sequencing
- Antibody expression in *E. coli*
- Antibody and scFv purification
- Immunoassays (ELISA, flow citometry)
- Surface plasmon resonance
- Confocal microscopy
- Gastrin-neutralization assays and cell cultures
- Affinity maturation methods
- Antibody humanization methods

Achievements made

- 57 gastrin-specific mouse monoclonal antibodies
- 19 gastrin-specific human scFv
- 3 gastrin-specific mouse scFvs
- 27 unique and different amino acid sequences corresponding to the variable regions of gastrin-specific antibodies and scFvs
- Several *in vitro* affinity matured scFvs
- 1 humanized mouse monoclonal antibody
- Gastrin-specific antibodies able to inhibit gastrin-derived proliferation
- Four manuscripts (1 published, 1 submitted and two more in preparation)



PARTNER 3. UNIVERSITE LOUIS PASTEUR (ULP)

Objectives and starting point

The contribution of **ULP** to the « Binding Gastrin » project was to use surface plasmon resonance (SPR) for characterizing the binding properties of molecules produced at **CNIO** (antibody and antibody fragments) and **PS** (binding bodies) . Fig. 1 summarizes the information retrieved from SPR experiments for the different classes of molecules. This work required extensive exchange of material (scFvs, gastrin, bbs) and information (sequences, SPR results) between **CNIO**, **PS** and **ULP**.

Methodologies employed

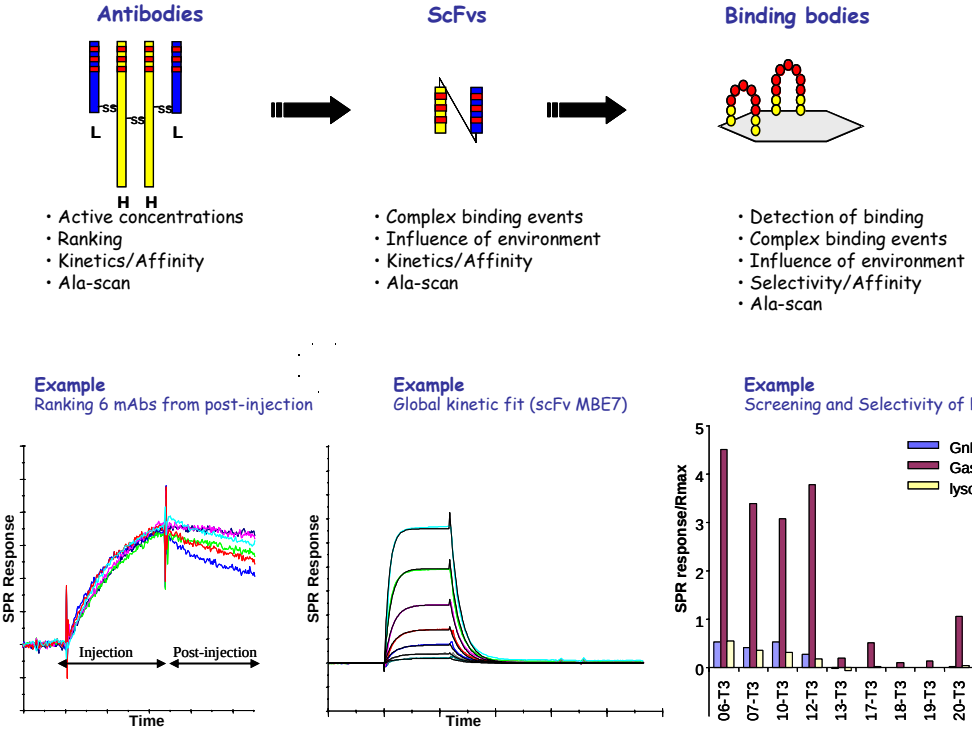
- Binding-affinity measurements (K_d, SPR)
- Active concentration measurements (SPR)
- Studying binding kinetics (k_{on}, k_{off}, SPR)

SPR work performed at ULP demonstrates that

- anti gastrin scFvs selected at CNIO were improved from microM to nanoM affinities
- their inhibition potential in cellular assays performed at UMCU correlates with their kinetic off-rate
- regions recognized on gastrin by the scFvs always include a tryptophan (W4 or W14) as essential residue, together with a negative charge (E1 or D16), essential in 12/15 cases
- bbs designed at PS were improved from aggregated material with non measurable binding strength to monodisperse binders with gastrin affinity in the 100-500 microM range
- a constrained structure is required for the detection of gastrin binding
- the synthetic bbs are selective for gastrin
- W4 is essential for gastrin recognition by 3 of the bbs

Summary table of affinity data

Molecules	**K_D1' or K_D' range (10^{-8} M)**	**k_{off} or k_{off}1 range (10^{-3} s^{-1})**
mAbs	0.02-2.5	0.6-20
Human Tomlinson scFvs	100-1.000	Not measurable
Human Mehta scFvs	0.2-20	5-100
Mouse scFvs	2-1.000	0.4-600
Binding bodies	10.000-50.000	Not measurable



PARTNER 4. ALGONOMICS NV (AN).

Objectives and starting point

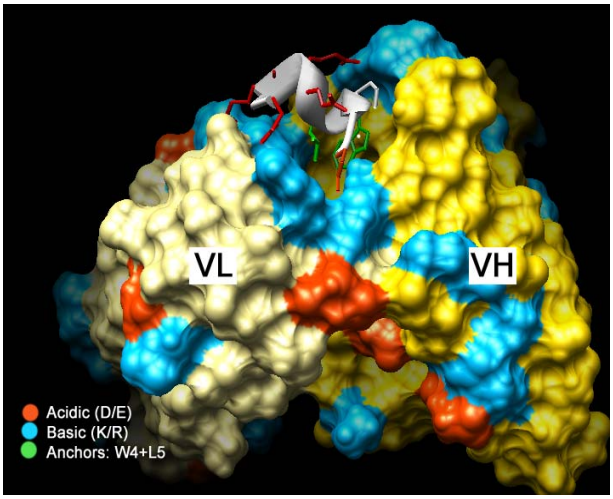
The main contribution of **AN** in this project was to provide a structure-based analysis of experimental data on antibody/gastrin complexes and to apply this information to the design and characterisation of binding body constructs. **AN** was involved in a design cycle comprising the steps of (i) retrieving a set of antibody Fv sequences, (ii) building 3-D models for each, (iii) analysing the features driving gastrin binding, and (iv) formulating proposals for optimised antibodies and/or binding bodies. For this purpose, diverse molecular modelling techniques were at disposal, whereas others had to be developed.

Methodologies employed

- Diverse molecular modelling techniques incorporated in the Brugel™ package, including
  - sequence and structure alignment methods
  - selection and assembly of 3D-structural templates
  - energy optimisation method (molecular mechanics)
  - structure variation analysis (molecular dynamics)
  - side-chain optimisation (proprietary FASTER algorithm)
- Expertise in Ab structure building and engineering
- A proprietary method for ligand/receptor affinity scoring

Achievements made

- Elaboration of an antibody variable domain structural database
- Development of a novel method for semi-automated scFv modelling
- Modelling of over 40 specific scFv structures
- Development of a new protocol for peptide docking to scFv structures
- Docking of gastrin to 2 selected scFv's in different binding modes
- Homology modelling of 6 scFv/gastrin complexes
- Design of "mini-antibodies" composed of selected H3-L3-H1-H2 fragments (12 variants)
- Design of a CCK-A receptor-derived construct with proven specificity for gastrin
- Various structure-based proposals for antibody affinity maturation (yielding sub-nM affinity)
- Full rationalisation of affinity-matured scFv's



3-D model of the complex between the gastrin peptide (ribbon) and the scFv ELISA-E1 (surface envelope). Gastrin is bound via its central fragment adopting a helical structure. Various Glu residues (red sticks) are electrostatically attracted by positively charged residues on the scFv surface (blue). Trp 4 and Leu 5 of gastrin (green sticks) form a pair or hydrophobic anchors that are deeply buried in the complex.

PARTNER 5. PROTEOMIKA S.L. (proteom)

Objectives and starting point

Proteomika has applied its knowledge and experience in protein arrays to the high throughput screening for the analysis of binding bodies. Our participation in the project has included two stages:

- First, optimisation of materials and methods has been carried out using models as different types of binding bodies, mAbs and scFvs.
- Second, once set up the protocols for each approach, the screening of binding bodies, scFvs and mAbs has been made.

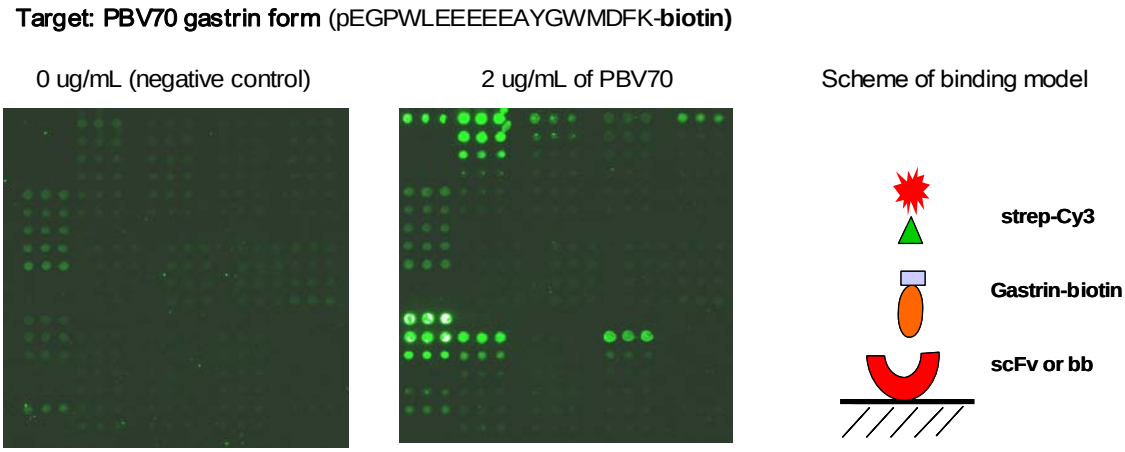
Methodologies employed

Analysis of binding has included the optimisation of specific tools and conditions in the spotting system (mechanical conditions of printing, printing buffer, concentration of capture probes and optimal slides) and array incubation conditions (blocking step, target concentrations and detection conditions). For this purpose, several intermediate models have been used as anti-lysozyme Abs and anti-GnRH binding bodies, maybe not ideal for gastrin binding but fine models to set up the conditions in the arrays. Two approaches have been studied:

- direct arrays (detection of labelled target)
- reverse-phase arrays (detection of printed non-labelled target)

Achievements made

- A series of gastrin binders, including scFvs (15), mAbs (4) and binding bodies (18 non-labelled and 3 labelled), have been screened for target binding in arrays.
- The best binding probes have been detected in each case: BIOT4E7, DTA3 and DTE6 for the scFvs tested; the mAbs 198CAB (against C-terminus) and 119EB1 (against N-terminus) where an ELISA type detection method has been developed; and BB189-T3, BB193-T3 and PDW16-T3 non-labelled binding bodies, and PDE38 and PDK12 as biotin-labelled binders.
- Some of the scFvs and mAbs can be considered as positive binders of gastrin once printed on the slides and that could be used for clinical applications.
- The labelled binding bodies tested in solution using reverse-phase arrays recognize and bind to printed gastrin. However, the majority of binding bodies studied did not bind efficiently to gastrin when analysed as capture molecules in direct arrays. Thus, it may be that immobilization on the array can affect to the structure-function of the studied molecules.



Fluorescent detection of binding of gastrin to immobilized probes including mAbs, scFvs and bb´s in protein arrays.

PARTNER 6. UNIVERSITY MEDICAL CENTER UTRECHT (UMCU)

Objectives and starting point

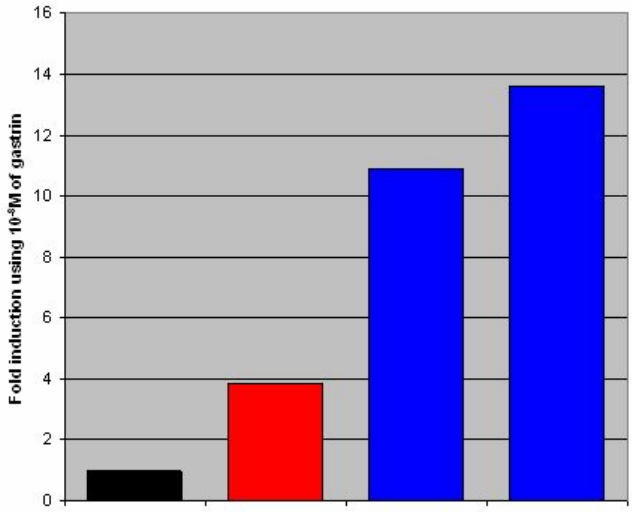
The role of **UMCU** in this project was to 1) develop an *in vitro* and an *in vivo* gastrtin bioassay and 2) to evaluate the gastrin neutralizing potential of gastrin anti-/binding bodies in these bioassays.

Methodologies employed

- Gastrin-induced calcitonin secretion from human MTC cell lines
- Gastrin-binding to cell lines expressing the gastrin receptor (ligand-binding assay)
- Gastrin-mediated expression of a luciferase reporter gene, under control of a gastrin-inducible c-fos promoter, in transfected pancreas and colon cancer cell lines
- Gastrin antibody-mediated inhibition of gastrin-induced c-fos-luciferase expression
- Alanine-scanning of amino acids in gastrin, relevant for binding to the gastrin receptor
- Gastrin-induced calcitonin secretion from MTCs in transgenic mice *in vivo*
- Gastrin-induced gastric acid secretion from the stomach of rats equipped with a gastric fistula *in vivo*

Achievements made

- Gastrin does not significantly and reproducibly induce calcitonin secretion from 8 human MTC cell lines tested (whereas it does induce calcitonin secretion from human MTCs *in vivo*: "C-cell stimulation test" used in clinical practice)
- Radio-labeled gastrin binding to human MTC cell lines is not diminished by a large excess of unlabelled gastrin
- Gastrin shows a reproducible, dose-dependent increase in c-fos-luciferase activity in transfected pancreatic and colon cancer cell lines (*in vitro* gastrin bioassay)
- Gastrin mAbs and scFvs show an inhibition of gastrin-induced c-fos luciferase activity in transfected rat pancreatic cancer cells
- Identification of specific amino acids in gastrin, required for binding to the gastrin receptor
- Gastrin does not induce secretion of calcitonin from mouse MTCs *in vivo*
- Intravenous gastrin injection in rats equipped with a gastric fistula increases gastric acid output (*in vivo* bioassay)



Increase in c-fos-luciferase activity by 10^{-8} M gastrin in pancreatic cancer cells (red column) and colon cancer cells (left blue column). The gastrin response was even stronger in colon cancer cells stably transfected with the human gastrin receptor (right blue column).