PROJECT FINAL REPORT

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Antibody Derivatives As Molecular Agents for Neoplastic Targeting

Collaborative Project (small or medium-scale focused research project)

4.1 Final publishable summary report

4.1.1 Executive summary

Monoclonal antibodies represent the largest and fastest growing class of Pharmaceutical Biotechnology products, as measured in terms of cumulative sales and of number of products in clinical development. Antibodies can be used to deliver bioactive molecules (drugs, cytokines, photosensitizers, radionuclides, fluorophores etc.) to the tumor environment, thus enabling molecular imaging applications or therapeutic interventions which spare normal tissues. The antibody-based targeting of tumor neo-vasculature is particularly attractive, because of the dependence of cancer on new blood vessels and because of the accessibility of these structures from the blood-stream. The formation of new blood vessels ("angiogenesis") is a rare process in the healthy adult, mainly confined to the female reproductive system.

In the ADAMANT Project, we have developed and implemented state-of-the-art methodologies for the identification and validation of proteins which are preferentially expressed in the tumor neo-vasculature and stroma. These markers of angiogenesis have been used for the generation and extensive characterization of human monoclonal antibodies, whose *in vivo* tumor targeting properties have been investigated by quantitative biodistribution analysis and by imaging studies in tumor-bearing mice. In addition, the human monoclonal antibody F16, labeled with the radionuclide iodine-124, has been studied in a Phase 0 clinical trial for the positron emission tomography (PET) imaging of patients with cancer.

The newly developed monoclonal antibodies have been chemically modified to serve as delivery vehicles for bioactive payloads (cytotoxic drugs, radionuclides or cytokines). The therapeutic potential of the resulting armed antibodies has been studied, both alone and in combination with other drugs, in rodent models of cancer. This has led to the development of novel biopharmaceutical agents which could eradicate tumors that are not cured by conventional pharmacological interventions. Some of the newly developed products and combination treatments are now being studied in clinical trials.

The ADAMANT Project has led to an improved understanding of the molecular composition of the tumor neo-vasculature and of the tumor stroma. Furthermore, the Project has allowed the development of novel anti-cancer products, stimulating industrial activities in Europe and leading to the implementation of novel therapeutic concepts and of new clinical trials.

4.1.2 Summary description of project context and objectives

Within the ADAMANT Project, the development of innovative antibody-based pharmaceutical products targeting the tumor neo-vasculature and the tumor stroma has required innovation in three main experimental parts.

In the first part of the Project, we have aimed at providing a comprehensive analysis of proteins which are preferentially expressed in the neo-vasculature of solid tumors and of lymphomas. To this aim, we have used a chemical proteomics methodology, invented by members of the ADAMANT Consortium, for the identification of accessible vascular markers in normal tissues and at sites of disease. The technology relies on the terminal perfusion of animal models of pathology (e.g., tumor-bearing mice) with a reactive derivative of biotin, which covalently modifies accessible proteins. Alternatively, the biotinylation reaction can be performed by *ex vivo* perfusion of surgically resected human organs with cancer. Biotinylated proteins can be purified on streptavidin resin in the presence of stronge detergents, digested and the resulting tryptic peptides separated by HPLC and analyzed by mass spectrometry in the presence of internal standards for quantification purposes. This research activity has led to the development of an "Atlas" of vascular proteins expressed in various tumor types, such as solid and metastatic tumors or lymphomas. In addition, a transcriptomic study of gene expression in endothelial cells under different angiogenic stimuli have revealed a complementary set of candidate markers of angiogenesis. The most promising target proteins have been validated using immunochemical techniques, in part thanks to the development of new monoclonal antibodies.

In the second portion of experimental activities, human monoclonal antibodies have been isolated from large phage display libraries. These new reagents have been extensively validated using immunochemical techniques (e.g., immunohistochemistry, immunofluorescence) in tumor specimens and normal tissues. Furthermore, many new antibodies have been studied using nuclear medicine techniques, such as imaging or biodistribution studies in tumor-bearing mice.

In the third set of experimental activities, novel biopharmaceuticals have been developed by arming the most promising recombinant antibodies with three main classes of bioactive payloads: (i) drugs with cleavable linkers; (ii) radionuclides; (iii) cytokines. Novel cytotoxic drugs have been developed (e.g., tubulysin or dolastatin analogues), which incorporate chemical handles for the site-specific coupling to recombinant antibodies. Similarly, novel procedures have been invented and/or implemented for the radiolabeling of antibodies for imaging and therapy applications. The ADAMANT Consortium has also systematically investigated the field of antibody-cytokine fusion proteins ("immunocytokines"), which have revealed impressive therapeutic activities, especially when used in combination with other judiciously selected pharmaceutical agents. Some of the most impressive curative effects, which have resulted in the complete eradication of aggressive tumor types in rodents, include the combination of IL2-based immunocytokines with taxanes or with monoclonal antibodies (e.g. rituxan) which are already used in clinical practice.

The ADAMANT Project has also included accompanying translational activities, to facilitate the implementation of the newly developed products and methodologies in the clinical practice. An immuno-PET Phase 0 clinical trial for the imaging of cancer patients with the F16 antibody (specific to the alternatively spliced A1 domain of fibronectin) labeled with ioine-124 has been started. Furthermore, the Consortium has collaborated for the preparation of clinical trial protocols and for the evaluation of clinical results with new biopharmaceuticals targeting the tumor neo-vasculature and stroma, thus contributing to an increased awareness of the opportunities and problems associated with this novel class of pharmaceutical agents.

4.1.3 Description of the main S&T results/foregrounds

Identification of new tumor-associated antigens, accessible and selectively present in human primary cancer lesions and metastases, by *in vivo* and *ex vivo* chemical proteomics

MILESTONES

- Characterization of accessible proteins in different primary malignancies and associated metastasis
- Development of chemical proteomic methods to study the accessible proteins in human tissues

OVERVIEW

A significant limitation of the most currently applied anti-cancer treatments is related to their toxicity. Indeed, chemotherapy relies on the expectation that anticancer drugs will preferentially kill rapidly dividing tumor cells. Unfortunately, they also target normal cells, particularly cells from tissues with high turnover, such as hematopoietic progenitors and epithelial intestinal cells. This toxicity limits both the treatment duration and the deliverable drug doses, which significantly restrict the therapeutic efficiency needed to obtain and maintain a complete remission. One of the most attractive strategies for the development of new, efficient, and selective treatments is the delivery of targeted bioactive molecules (e.g. cytokines, radioactive isotopes, cytotoxic agents) selectively to the tumor microenvironment. This strategy is based on ligand binding with high affinity and specificity to target molecules, over-expressed at disease sites. Following this rationale, the discovery of protein targets readily accessible through the bloodstream and selectively over-expressed in pathological tissues has become a major research objective. Indisputably, this group of molecules has a high potential to serve as innovative tools for effective imaging and targeted cancer therapy approaches. Ideally, reachable cancer proteins (accessible biomarkers) of high therapeutic value should be expressed solely by the malignant tumor and be at least not accessible in normal tissues. In the frame of the ADAMANT project we have embarked on the development of suitable pre-analytical methods to study accessible proteins in human tissues. Following this Members of the Consortium have applied the newly developed technologies in order to characterise a variety of primary malignancies and associated metastasis both in human clinical samples and suitable in vivo models.

Along these lines, the original methodology was developed in a previous EU collaborative project (STROMA 2004-08) by ETHZ in collaboration with IRFMN who applied a chemical proteomic strategy, based on the terminal perfusion of tumor-bearing rodents [Rybak et al, Nature Methods, 2005] with reactive ester biotin labels and subsequent labeled-protein recovery including mass spectrometric analysis. The major aim was to establish an atlas of vascular proteins in health and disease. This technology was further improved in collaboration between ETHZ and ULG, yielding novel methods where first human tumor bearing organs were ex vivo perfused [Castronovo et al, Molecular and Cellular Proteomics, 2006] and latter tumor biopsies were in vitro labeled [Castronovo et al, Proteomics, 2007] in order to gain access to potentially targetable proteins.

In the frame of the ADAMANT project the technique has improved several times mostly in terms of being more quantitative [Fugmann et al., Proteomics, 2010; Strassberger et al., Proteomics, 2010]. Recently, the technology has been further refined by involving glycosylated proteins, in addition to biotinylated ones, in order to complement the repertoire of potentially accessible biomarkers [Turtoi et al, Journal of Proteome Research, 2011].

Once the suitable methodologies were established the partners have moved to characterize a broad range of malignancies and to establish the map of potentially accessible tumor biomarkers. suitable for targeted therapy. The research was primarily directed on colon, liver (metasyasis), pancreas, breast, bone (metastasis) and glioblastoma tumors from patients.

Breast Cancer. The above recent developed method which combines biotinylation with glycoproteomics has been applied on the breast cancer tissue: a novel biomarker has been discovered (CD276) and validated in more than 30 individual cases.

Bone metastasis. The investigation of a breast tumor associated bone metastasis was finalized. This study had the unique opportunity to examine bone metastasis and the corresponding breast cancer primary lesion obtained simultaneously from a fresh autopsy performed on a patient who died from disseminated breast cancer. The accessible protein biomarkers were identifying using the previously developed combinatory procedure of biotinylation and glycoprotein extraction. Altogether, twenty nine of the potential biomarkers were found uniquely expressed in the primary breast cancer while 27 proteins were detected only in the bone metastasis lesions alone. In particular several proteins belonging to small leucine rich proteo-glycans, thrombospondin and integrin families were found up-regulated in the primary breast tumor.

Pancreatic Cancer. The proteomics technology has been applied to characterize pancreas ductal adenocarcinoma (PDAC) and its accessible protein biomarkers. Mass spectometry (MS) analysis revealed 484 differentially expressed proteins, of which 84 were evaluated as potentially accessible. Of these, 11 selected candidates were confirmed by western blot and Multiple Reaction Monitoring (MRM)-MS analysis. Transforming growth factor beta-induced (TGFBI), latent transforming growth factor beta binding protein 2 (LTBP2) and asporin (ASPN) were further investigated employing immunohistochemistry. They were found to be significantly expressed in a large group of clinical PDAC samples compared to corresponding normal and inflammatory tissues. In conclusion, TGFBI, LTBP2 and ASPN are novel, overexpressed and potentially accessible proteins in human PDAC.

Glioblastoma multiforme. Human glioblastoma specimens as well as tumors developed in nude mice (xenografted human glioblastoma U373 and T98G cells) along with normal mouse brains were biotinylated. These samples were analyzed with the above described techniques and mutually compared. Further to this an easy and cost effective method to validate the potential accessibility of tumor antigens *in svivo* has been developed . This assay is conducted with xenografted U87 human glioblastoma cells on the chicken egg choriallantoic membrane (CAM). Antibodies against suitable targets can be injected intravenously in the CAM vasculature and visualized ex-vivo using immunofluorescence. The current study has highlighted several known and novel proteins implicated in the human glioblastoma: tenascin-C, CD44, collagen-VI-A1, sparc-like-1 and prosaposin. Immunohistochemical validation experiments confirmed the expression of these proteins in large series of human glioblastoma cases. Specifically anti-CD44 and anti-collagen-VI-A1 have been successfully injected/ validated in the CAM/ U87 model.

Colorectal Cancer. *Ex vivo* perfusions of surgically resected human colon cancer (CRC) using biotin derivative, followed by comparative mass spectrometry-based proteomic analysis of the labeled proteins, revealed quantitative differences between normal and cancer colon. Sixty-seven of the total 367 proteins identified were found to be preferentially expressed at the tumor site. Human monoclonal antibodies against 2 potential tumor targets, NGAL and GW112, were generated and their selective expression in cancer colon and not or barely in healthy tissues proved [Conrotto et al, International Journal of Cancer, 2008].

Liver metastasis. In over the half of CRC patients a metastatic disease is present at the time of diagnosis, reducing the 5-year survival to less than 5 %. One of the most common sites of metastasis is liver, characterised as asymptomatic until the disease is well progressed. Therefore an effective treatment and/or early diagnosis modality for the CRC liver metastasis would make a significant difference for a large population of patients. Accordingly, we aimed at investigating whether the metastasis displays a homogeneous distribution of certain biomarkers or/and some biomarker are only found in distinct regions of the lesion. Consequently, we have ex-vivo biotinylated accessible proteins from several CRC-liver metastases and divided the specimen in 4 zones: normal, peri-tumoral, tumor-rim and center. The proteins were affinity purified and analyzed for each zone separately using mass spectrometry. In total over 1500 unique proteins were statistically divided into six patterns of expression. Approximately 1/3 was expressed solely in one of the 4 zones. A further 1/3 was found in all zones. Remaining proteins were present in 2 or 3 regions studied. Several known and new markers have prompted our interest among which: periostin (POSTN), transforming growth factor beta-induced (TGFBI) and latent transforming growth factor beta binding protein 2 (LTBP2).

Next, the found antigens were validated in a mouse model of CRC liver metastasis. Mice liver metastasis and corresponding normal tissues were biotinylated and analyzed by MS. The investigation revealed 922 mouse proteins present solely in the normal liver, 704 human proteins present in the metastasis and 248 proteins (mouse and human origin) found in common between normal mouse liver and mouse liver metastasis. Of the in common proteins 70 were up-regulated in the tumor. Finally we have assessed the potential accessibility of the identified proteins and found that 110 proteins qualify as differentially expressed and potentially accessible. The data were compared with results obtained from the analysis of human liver metastases and resulted in 64 proteins which were modulated both in the mouse model as well as in the human liver metastases. Further validation studies are ongoing.

Furthermore, an *in vivo* biotinylation of vascular structures by terminal perfusion was performed in three different syngeneic mouse models of CRC liver metastasis The recovery and mass spectrometric analysis of accessible biotinylated proteins in liver metastasis revealed quantitative differences in the expression of vascular proteins compared to the host organ. The preferential expression of ten markers at metastatic sites was confirmed by immunofluorescence analysis and, in the case of periostin, oncofetal fibronectin and angiopoietin-like 2 protein, by in vivo targeting experiments using radiolabeled antibody preparations [Borgia et al, Cancer Research, Cancer Research 2010].

Antibodes production and target validation

MILESTONES

- Generation and production of new recombinant human antibodies against tumour specific antigens
- In situ validation of antibody reactivity, specificity and target distribution in human tissue

OVERVIEW

Tumour vessel and tumor environment specific molecules can serve for therapeutic purpose and can be developed as targets for antibody based pharmacodelivery. An important precondition for their

possible future clinical exploitation is the assessment of the "novel" target distribution and of the specificity/reactivity of the newly produced antibody against it.
Following the discovery of novel tumor specific accessible antigen (s) a key task is the generation of antibodies and their deep investigation. Immunohistochemical staining methods were optimized and the antibody reactivity tested in a wide range of normal, tumorous and non-tumorous pathologies and the distribution of the accessible antigen evaluated. As result several newly produced human recombinant antibodies could be characterized and new putative tumor specific targets could be validated, as a prerequisite for the preclinical studies aimed at assessing therapeutic efficacy of the conjugated antibodies (described in the following paragraphs).

Target selection and first level of validation

New candidates for tumour associated vascular markers were selected by from transcriptomic studies in human cancer tissues, isolated endothelial cells as well as in high and low VEGF expressing ovarian carcinoma xenografts. The selected candidates include ADAM 23, GPNMB, FAP and RGS5 (**Figure 1**). Tumour stroma restricted antigens like MG50, periostin, uPA, MMP1, 2, and 3, carbonic anhydrase IX, ED-A and ED-B fibronectin and also tenascin-C splicing variants were selected for antibody production.

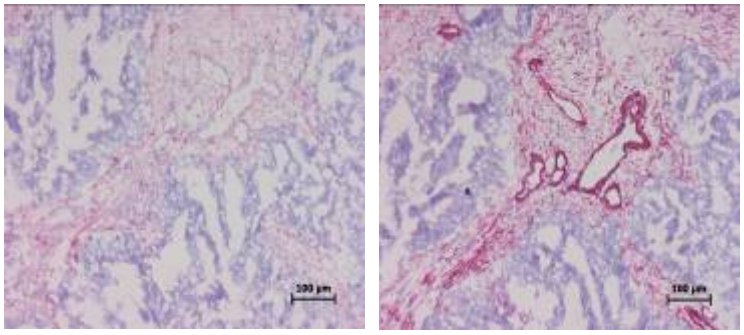

Figure 1: *Prostatic Adenocarcinoma – immunostaining for two novel tumor stoma/vascular targets*

Antibody production

Philochem has implemented the Transient Gene Expression (TGE) as a standard methodology for the simple, versatile and fast (from months to weeks) generation of proteins against which antibodies should be raised (**Figure 2**).
Using this method several antigens could be expressed.
For recombinant antibody production two new synthetic human antibody libraries (named PHILO1 and PHILO2) were cloned to broaden the epitope recognition in respect to the previously characterized antibody library (ETH2-GOLD). Additionally, a fully murine antibody library (PhiloTOP) was produced allowing the production of murine antibodies against markers which are not immunogenic in the mouse (e.g. EDB). Such antibodies serve as research tools in order to test long term therapy effects of fully murine proteins in immunocompetent mice.

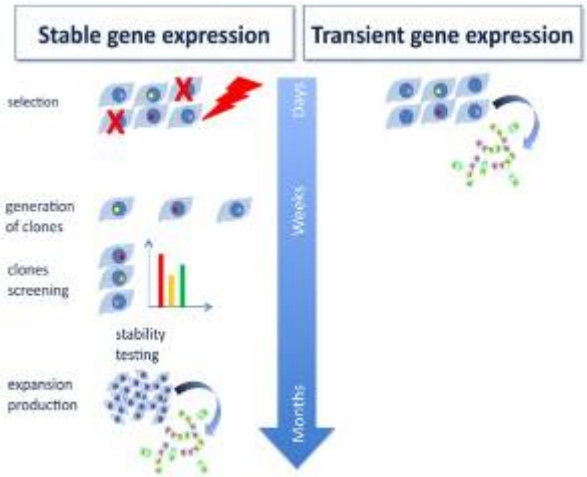

Figure 2*: Transient Gene Expression (TGE)*

With the aid of all these technologies/methodologies, recombinant antibodies against the selected stromal and vascular antigens were successfully produced.
Among them are high affinity antibodies against human ED-A and ED-B fibronectin, MG50, periostin, uPA, human carbonic anhydrase IX (CAIX) and the vascular marker of B-cell lymphoma BST-2 , as well as against murine matrix metalloproteinases 1, 2, and 3 (MMP1, 2, and 3) and EDB-domain of fibronectin [Pfaffen etal, Exp Cell Res. 2010a; Eur J Nucl Med Mol Imaging. 2010b; Schliemann et al, Blood 2010]

Target distribution and antibody reactivity validation

The tissue distribution of these putative target structures was investigated in detail. For a variety of the markers a nice tumour or tumour vessel restricted occurrence could be proven. The tissue reactivity of the recombinant antibodies L19, F8, F16, G11 and D11 directed against fibronectin and tenascin-C variants were comparatively examined. For histological analysis a thyramid based amplification system was introduced leading to sensitive and reproducible immunofluorescence staining results for the biotinylated recombinant antibodies. Fluorescence double and triple staining procedures also combining different recombinant antibodies were designed to investigate putative target structures in relation to vessels, stromal components, tumour cells and basement membrane structures as well as in relation to tumour type, subtype, malignancy grade and progression (**Figure 3**).

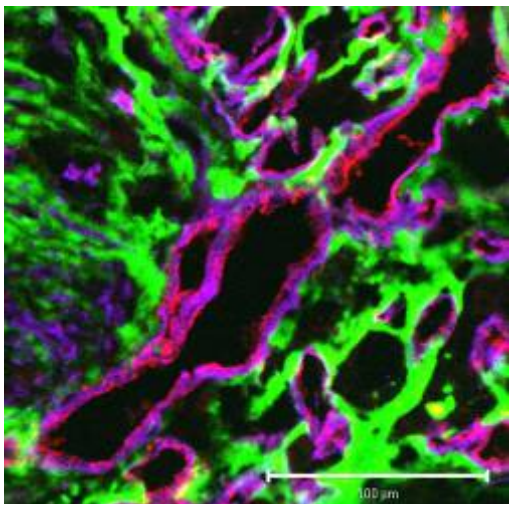

Figure 3*: Triple fluorescence staining for tenascin-C A1 domain (green), laminin a4 chain (blue), and endothelial cells (red) in a clear cell renal cell carcinoma.*

It could be demonstrate that fibronectin and tenscin-C variants show different expression patterns discriminating tumours with wide stromal deposition from tumors with a restricted vascular deposition. Moreover, the incorporation of fibronectins or tenascins in the blood vessel wall of the tumor was different, too. These observations are of special interest for the selection of candidate tumours for treatment with fibronectin or tenascin-C antibodies and for individualized therapy strategies. Clinically valuable expression patterns could be described for melanoma, lymphoma, lung carcinoma and renal cell carcinoma. For the first time, the spatial relation of vascular fibronectin and tenascin-C deposition in relation to vascular BM was defined by laser scanning microscopy colocalization studies showing a stratified organization of the vessel wall matrix [Berndt et al, Histochem Cell Biol. 2010]

With respect to the preclinical studies aimed at assessing the efficacy of the antibodies as therapeutics, the reactivity of several recombinant antibodies against fibronectin and tenascin-C isoforms as well as against MMPs, uPA and CA IX was investigated in human tumor xenografts and in syngeneic mouse tumors, with particular focus on renal cell carcinoma, ovarian cancer, melanoma, and lymphoma (**Figure 4**) [Ahlskog et al, Br J Cancer, 2009; Coltrini et al, The J of Pathology, 2009]. Tumours strongly vary in their stromal and vascular expression of antigens recognized by the recombinant antibodies depending on the tumour type, the mouse strain (immunological background) and the species reactivity of the antibodies. Experimental tumours reflecting the results obtained with the clinical specimens were candidate to undergo experimental targeted therapy.

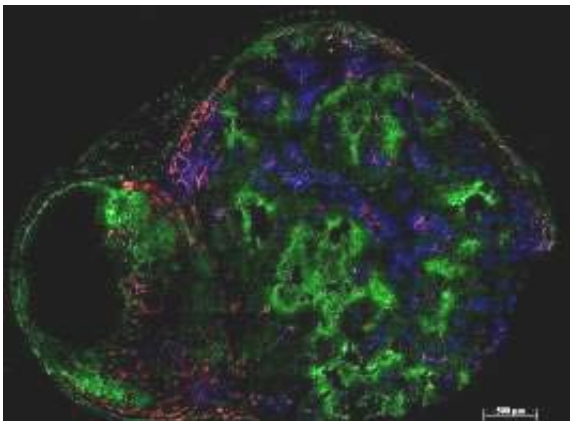

Figure 4: *Distribution of carbonic anhydrase IX (CA IX) in a colorectal tumour model (green), blood vessels (red), and perfusion (blue).*

Target distribution in non neoplastic pathologies

Targeted therapy of human neoplastic diseases using the recombinant antibodies F16, F8 and L19 (the best candidates for clinical development) might cause systemic side effects in healthy organs or in other non-neoplastic pathologies if there is an expression of the antigen/target molecules. Since no reactivity could be appreciated by analysing the collection of healthy tissue available commercially, it was a central task to analyse non-neoplastic pathologies.

Because of the high incidences of both neoplastic and cardiovascular diseases there are high co-morbidity rates, the reactivity of the above mentioned antibodies were assesses in different cardiac diseases to evaluate the risk of side effects and to possible identify new fields of potential diagnostic or therapeutic use.
Studies were focused on evaluating the antigens recognized by the F16, F8 and L19 antibodies in pathologies of the cardiovascular system.

F16 antibody stained areas of active tissue remodelling in atherosclerotic plaques (in both human and animal models) and may thus deserve to be exploited as a suitable building block for the development of radiopharmaceuticals for plaque imaging or for an antibody-based targeted delivery of therapeutic agents to atherosclerotic lesions.

Diseased human cardiac tissue of the right atrial auricle (RAA) and left ventricular septum (LVS) derived during cardiac surgery from patients with aortic valve stenosis (AVS) and/or coronary artery disease (CAD)were systematically analysed. A re-expression of F8 and F16 antigens cold be observed, together with a quantitative differences in the expression levels between AVS and CAD as well as in association to the grade of histological damage. Thus, F8 and F16 were suggested to be of potential interest for an antibody-mediated targeted delivery of diagnostic or therapeutic agents in human cardiac diseases with respect to anti-inflammatory or anti-fibrotic therapy strategies**.**

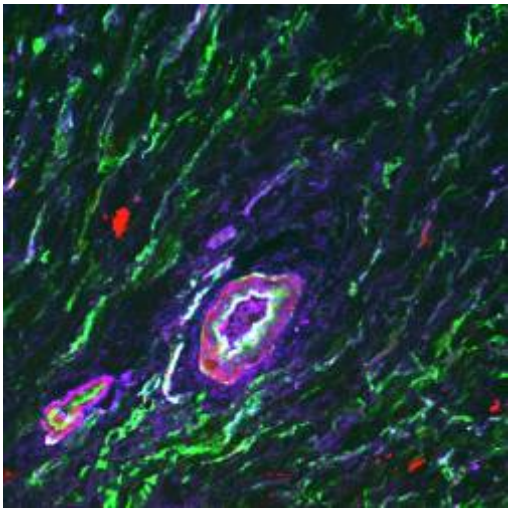

Figure 5: *Reactivity of the F8 antibody against ED-A$^+$ fibronectin (green) in cardiac allografts exhibiting signs of chronic rejection: spatial association to CAV and CIF (immunofluorescence triple labelling including ED-A$^+$ fibronectin (green), alpha-smooth muscle actin (blue) and B$^+$ tenascin-C (red))*

The reactivity of the recombinant antibodies were investigated in a heterotopic rat heart transplantation model of chronic cardiac allograft rejection. A relevant reactivity was demonstrated for F8 but not for L19, F16, D11 and G11. Tissue reactivity revealed an extensive antigen expression (ED-A fibronectin) in heart allografts exhibiting signs of chronic rejection compared to healthy controls with clear spatial association to vessels showing cardiac allograft vasculopathy (CAV) and to areas of cardiac interstitial fibrosis (CIF) (**Figure 5**). Thus, confirming ED-A fibronectin as a promising marker of chronic rejection (CAV and CIF) and F8 antibody as a vehicle for targeted therapy strategies [Pedretti et al, Atherosclerosis 2010; Franz et al, J Heart Lung Transplant. 2011]

Radioimmonotherapy (RIT)

MILESTONES

- Highly selective tumour localisation of novel radio- or fluorescently labelled antibody conjugate
- Development of effective radioconjugates for pre-clinical PET
- Pre-clinical antitumor activity of a radioconjugate causing significant growth inhibition

OVERVIEW

The use of tumour-targeting antibodies to deliver therapeutics to the tumour site is a highly selective form of cancer therapy, reducing the side-effects frequently seen after conventional treatments while

increasing the specificity. The aim of this work package is to develop novel and effective agents, using antibodies that target either the tumour vasculature or the tumour cells to deliver radiation to the tumour (radioimmunotherapy [RIT]). The antibodies are delivered into the circulation, and can therefore reach sites in the body which are inaccessible to external beam radiation. Initial investigations, to confirm tumour specificity, are performed with fluorescently labeled antibodies.

Highly selective tumour localisation of novel antibody conjugates, and enhanced therapy by a combination of treatments

The antibody F8-SIP directed against ED-A fibronectin selectively targets tumour blood vessels, as can be demonstrated by multifluorescence microscopy. As shown in **Figure 6** the antibody (red) shows the same localization as the vasculature (green) over the whole tumour, indicating that the vast majority of blood vessels will be targeted for therapy, and when looked at in high power it has a highly specific perivascular distribution in colorectal tumours**.**

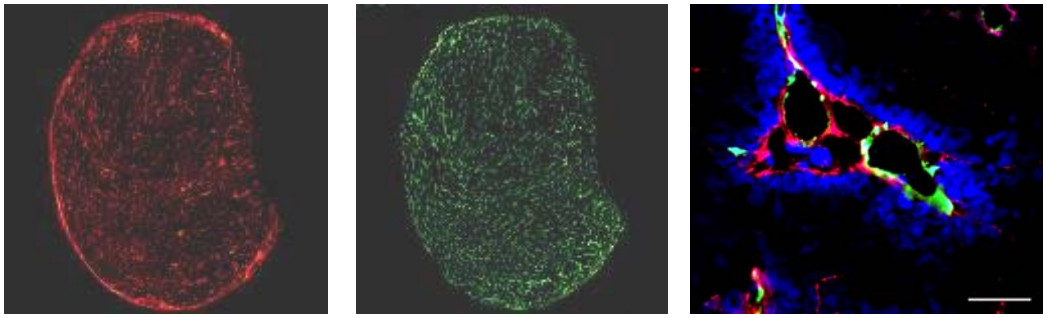

Figure 6 *Red: F8SIP, Green: blod vessels, Blue :perfusion*

The related antibody L19-SIP, when conjugated to radioactivity (^{125}I-L19-SIP), produces a significant therapeutic effect. We have been investigation ways to further increase this by using a combination of therapies, which treat different regions of the tumour. The vascular disrupting agent (VDA) combretastatin, currently in clinical trial, produces massive destruction of the centre of the tumour, but leaves a viable vascular rim which continues to grow. Combined therapy is therefore required to kill the whole tumour mass. We combined these 2 treatments, giving either radiolabeled antibody alone, or with combretastatin administered at 24 hours earlier, and imaged the effect of the VDA on radiolabeled antibody distribution across the tumour (**Figure 7**). In the antibody alone group the tumour was viable and the antibody was distributed heterogeneously throughout, with the hottest areas shown in red (**Figure 7A**). When combretastatin was also administered the tumours developed massive central necrosis, and the antibody was now concentrated within the remaining viable rim (**Figure 7B**) where it could be most afficacious. As the total radioactive dose to the tumour was the same in both cases, we have now achieved a complementary combined treatment with great therapeutic potential, which will form the basis of future radioimmunotherapy trials.

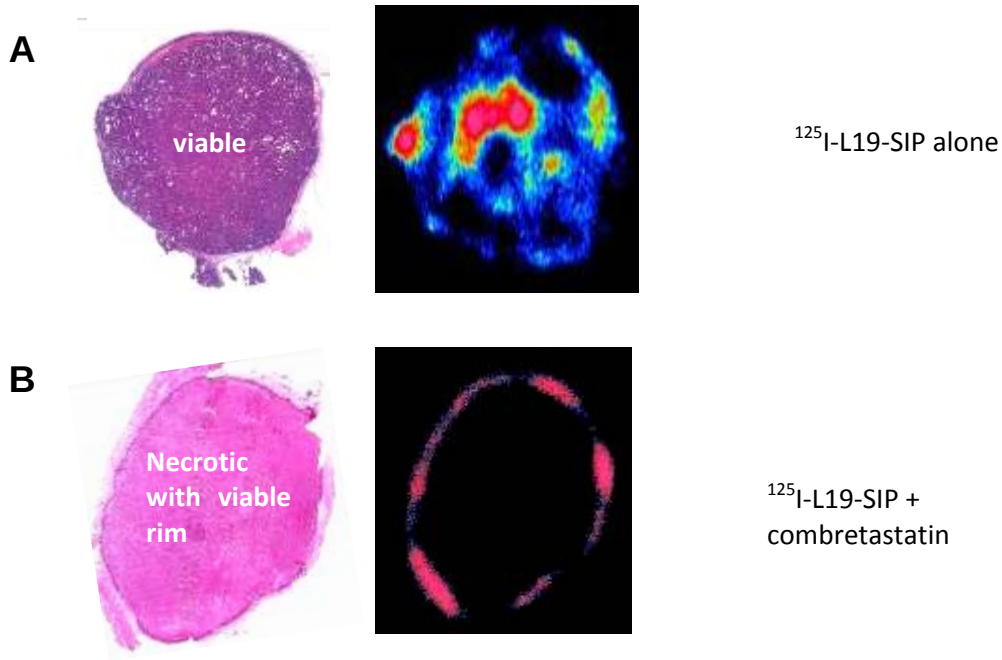

Figure 7 *The effect of combretastatin on the tumour distribution of L19-SIP antivascular antibody*

Development of effective radioconjugates for pre-clinical PET (immuno-PET)

There is a nuclear medicine requirement for non-invasive imaging of cancer patients in order to determine whether radiolabeled antibodies (radioimmunoconjugates) are successfully targeting, and being retained in, the tumour while clearing from normal organs, and also to determine target status. Procedures for development of these conjugates for single-photon emission computed tomography **(**SPECT) imaging are routinely available, but this is not the case for positron emission tomography **(**PET) imaging. We have therefore concentrated on developing standard procedures for producing suitable PET isotopes (positron emitters: ^{68}Ga, ^{124}I and ^{89}Zr), and conjugating them to tumour-targeting antibodies . For this purpose, new linkers have been developed, and logistics established for world wide distribution of both isotopes and linkers, while labeling protocols were published for dissemination of this so called immuno-PET technology. Several preclinical and clinical immuno-PET studies for the non-invasive *in vivo* assessment of target status and antibody distribution have been successfully carried out (**Figure 8**). Moreover, the potential of immuno-PET for quantitation of antibody distribution in patients has been evaluated. These achievements are of particular interest when using immuno-PET for selection of RIT candidates. This immuno-PET technology is now disseminated worldwide, and many leading academic centres and pharmaceutical companies use our technology in the (pre)clinical development of biotech products.

While nuclear imaging like SPECT and PET is particularly well suited for quantitative imaging of deep seated tumours, optical imaging using fluorescent dyes can provide complementary clinical potential for imaging superficial tissues. For this purpose a dualmodal nuclear/optical imaging probe is being developed, in which nuclear imaging is used for whole body imaging and optical imaging for local imaging. Since angiogenesis is an early event in tumor invasion, radioactively-fluorescently labeled dualmodal ligands directed against vascular tumor targets might be of diagnostic value and also a guide for RIT in future trials.

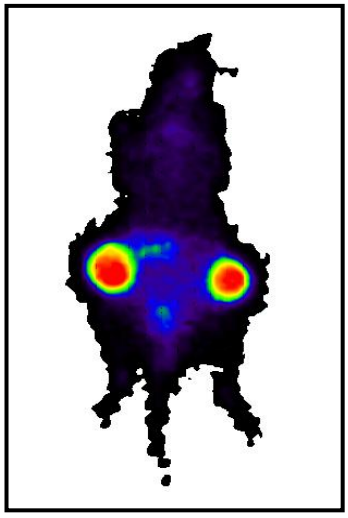

Figure 8 *Immuno-PET imaging of FaDu xenografts using ^{124}I-L19-SIP directed against tumour blood vessels. Red shows major activity.*

Radioconjugate producing significant tumour growth inhibition.
The major cause of death from colorectal cancer is the spread of disease to form liver metastases. We have therefore investigated the effectiveness of RIT in this clinically relevant site, using an antibody that targets a common marker of colorectal tumour cells called carcinoembryonic antigen (CEA). We initially attached a red fluorescent label to the antibody, and were able to demonstrate highly specific uptake in all tumour metastases, regardless of size, with none in the surrounding normal liver (**Figure 9**). We therefore attached radiation to the antibody for a therapy experiment, and found that we could significantly inhibit the growth of tumours in the liver, without concomitant toxicity [Dearling et al, Nucl Med Biol. 2009]. We also found that smaller liver metastases had far greater uptake of the therapeutic antibody than the larger deposits. This system is now being optimized for the treatment of small liver metastases in the clinic, where the prognosis is still very poor.

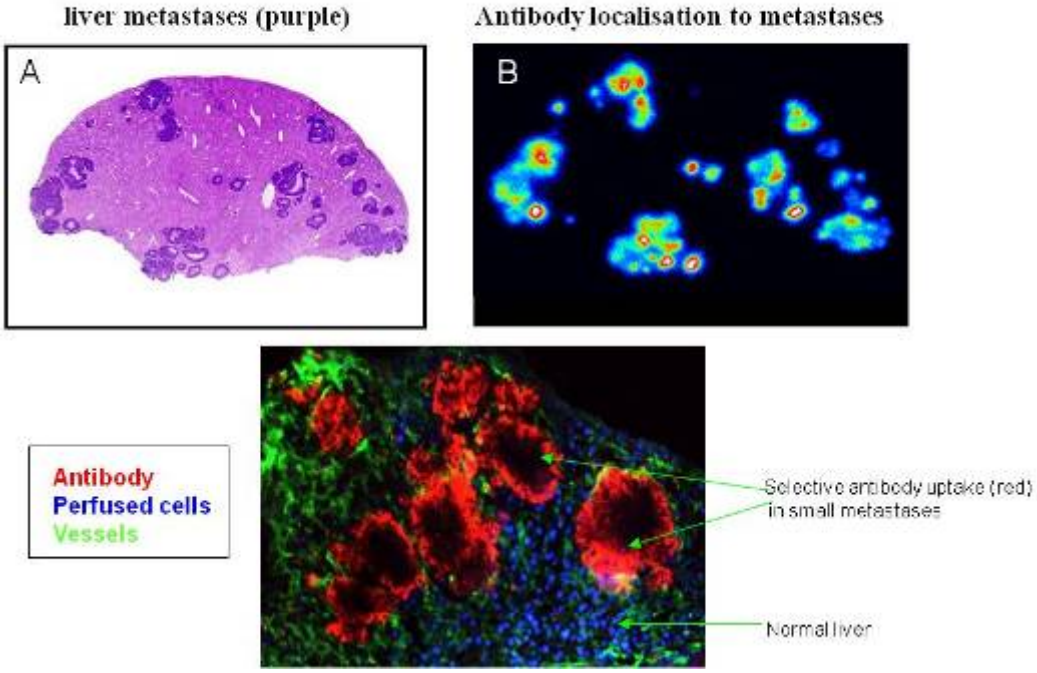

Figure 9 *Highly specific uptake of anti-CEA antibody into colorectal liver metastases, with none in normal liver*

Antibody drug conjugates

MILESTONES

- Generation of novel and effective antibody-drug conjugates, composed of either anti-tumor cell, anti-vascular or anti-stroma antibodies conjugated to tubulysins or dolastatin-15 analogues.

OVERVIEW

The availability of potent cytotoxic drugs having low-nanomolar or sub-nanomolar activity that can be conjugated to a monoclonal antibody through suitable chemical functions was the key for this part of the project. We decided to focus on three different classes of potent drugs, all having a natural origin:

tubulysins and dolastatins, which are both peptides, and vinblastin, which is an alkaloid. These molecules, which are unsuitable anti-cancer drugs as such because of their high toxicity and narrow therapeutic window, were synthetically modified to i) achieve the appropriate level of chemical and metabolic stability, ii) install chemical functions allowing for the attachment to the antibody via cleavable chemical linkers. The drugs might therefore be selectively released exerting a potent anti-tumor cell, anti-vascular or anti-stroma activity.

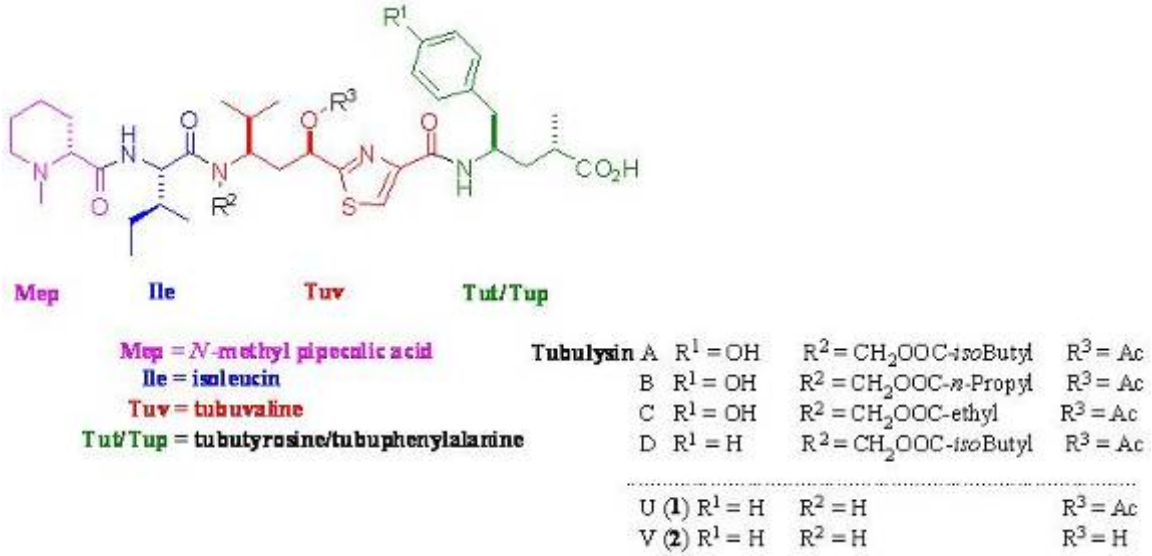

Figure 10 *Structure of the tubulysins derivatives suitable for conjugation to the antibody*

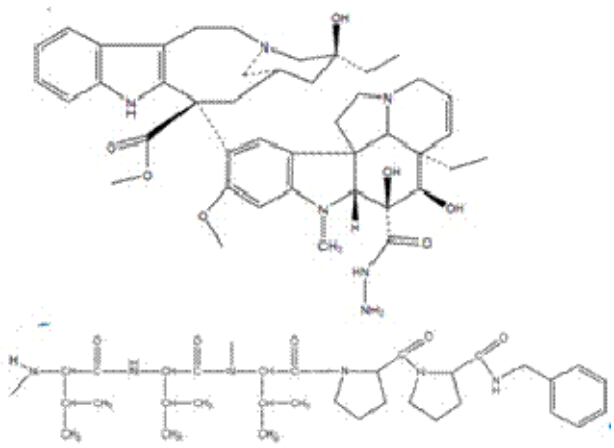

Figure 11*. Chemical structure of the derivatives of dolastatin-15 and of vinblastin used for antibody decoration.*

Almost 50 novel derivatives of these classes of highly potent compounds were synthesized, some of them in large quantities (hundreds of mgs) and high purity using conceptually new chemical strategies [Sreejith Shankar et al, Synlett 2011; patent EP 2292639 (A1) publication date 23-11- 2011]. Furthermore, these compounds were made available for biological studies on cancer cell lines and tumour animal models, and for experiments of antibody-functionalisation.

Some of the most potent tubulysins, dolastatins and vinblastin analogues were linked to human monoclonal antibodies or tumour homing peptides through disulfide or peptide linkers, thus obtaining several different conjugates [Palumbo et al, Br J Cancer 2011].

These conjugates, which were shown to have antibody-dependent anti-tumor effects in vitro, with IC50 values in the nanomolar range, were tested in vivo. Although these results are still preliminary, moderate retardation of tumor growth was observed with some of these conjugates, thus reinforcing vascular targeting antibody-drug conjugates as promising cancer therapy strategy.

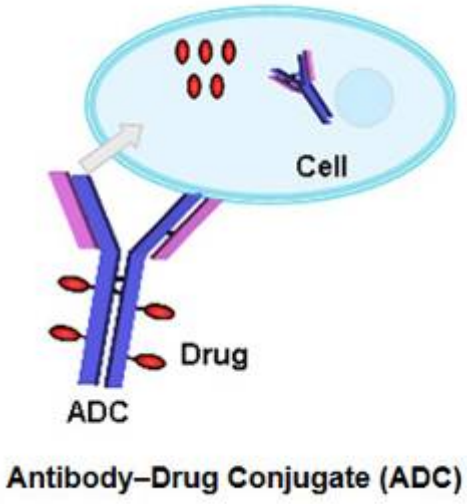

Figure 12. *Structure and mechanism of action of an antibody-drug conjugate.*

General recombinant antibody formats, which can be chemically modified with drugs and cleavable linkers at unique molecular positions, were also successfully engineered. For example, a method that enables specific and efficient conjugation of hydrazide-moieties to an IgG targeting the tumor neo-vasculature was successfully developed.

Immunocytokines

MILESTONES

- Cloning, expression, and *in vitro* characterization of novel immunocytokines
- *In vivo* testing of the therapeutic activity of novel immunocytokines

OVERVIEW

Many pro-inflammatory cytokines have been used for cancer therapy applications in clinical trials, but severe side effects have often prevented dose escalation to concentrations needed to induce cures. The fusion of cytokines with antibodies or antibody fragments, capable of selective accumulation at the tumor site, yields a new class of biopharmaceuticals (termed “immunocytokines”), which often displays superior therapeutic properties.

In the frame of the ADAMANT Project, we have produced and characterized several novel immunocytokines, based on IL2, IL7, IL10, IL12, IL17, IL18 and interferon-alpha as potent stimulator of the immune system. The resulting fusion proteins have been characterized *in vitro* (in terms of their pharmaceutical quality and activity) and *in vivo* (in terms of their tumor-targeting performance and therapeutic activity). Some of the tested immunocytokines (most notably those based on IL2, IL12 and IL15) have exhibited an impressive ability to inhibit tumor growth rate when used as single agent and to display superior performance compared to immunocytokine based on antibodies of irrelevant specificity in the mouse.

The antibodies used as fusion partners for the production of vascular tumor-targeting immunocytokines were F8 and L19 (specific to the alternatively-spliced EDA and EDB domain of fibronectin) and F16 (specific to the alternatively-spliced A1 domain of tenascin-C). As negative control, monoclonal antibodies specific to hen egg lysozyme (e.g., KSF), an irrelevant antigen in the mouse, were used.

The fusion proteins that were cloned, expressed and characterized (*in vitro* and *in vivo*) were many more than the four mentioned in the Project's Milestones [see also Publication List]. Below, we summarize some of the most significant findings of ADAMANT's research on immunocytokines.

The fusion protein F16-IL2 has exhibited a strong anti-tumor effect both in a subcutaneous and in an orthotopic model of glioblastoma multiforme. A microscopic analysis of tumor sections in mice treated with saline, temozolomide, F16-IL2 or a combination of temozolomide plus F16-IL2 has evidenced that the immunocytokine promotes a strong infiltration of leukocytes into the tumor mass [Pedretti et al, Br J Cancer 2010]

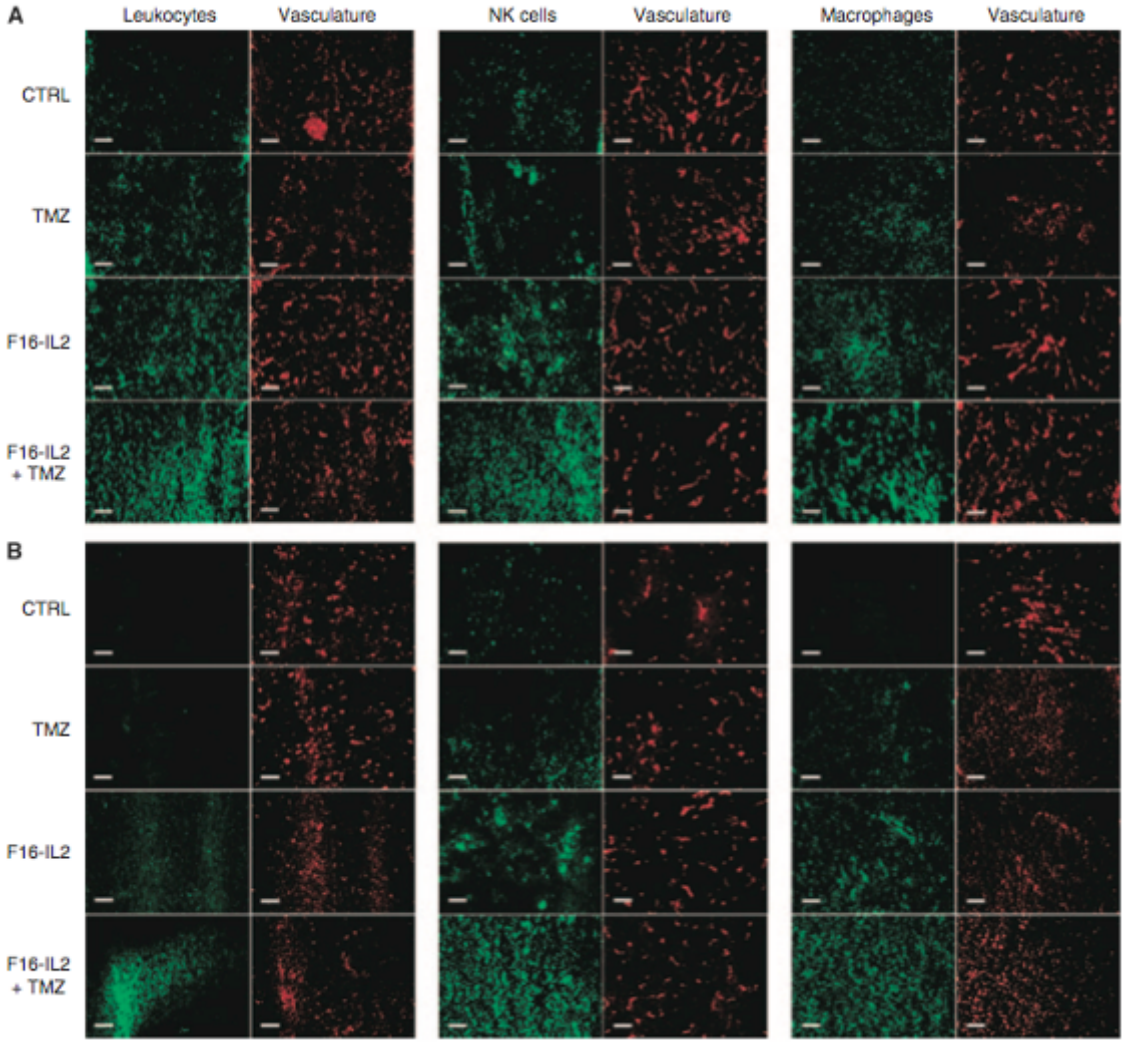

Figure 13: *Immunofluorescence analysis of tumour-infiltrating immune cells and of microvascular density in the subcutaneous (A) and intracranial (B) glioblastoma models, 24h after the third injection of therapeutic agents. The F16-IL2 + temozolomide treatment groups show the largest increase in the infiltration of leukocytes and in particular of natural killer cells and macrophages (serial tissue sections). Scale bars indicate 100 µm [adapted from Pedretti et al, Br. J. Cancer 2010].*

Interleukin-12-based immunocytokines have been investigated for many years by the ETH Zurich and Philochem scientists [Halin et al, Nature Biotechnology 2002]. This heterodimeric cytokine has traditionally provided challenges for protein expression (the N-terminus of the p40 subunit needs to be free for maximal biological activity), but also opportunities for the design of new formats. A novel disulfide-linked format of an immunocytokine consisting of the anti-EDA antibody fragment scFv(F8) and of human IL12 has recently been developed by the Philochem group and has been moved to GMP manufacturing. The protein displayed excellent pharmaceutical quality and impressive tumor-targeting performance in xenograft tumor models in rodents.

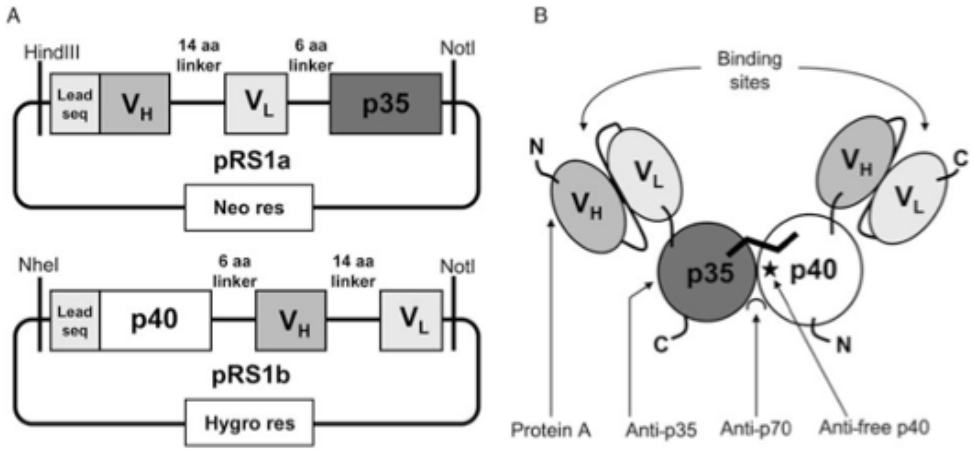

Figure 14 *Structure of F8-IL12. (A) Schematic representation of the cloning strategy of the F8-IL12 fusion protein. (B) Protein structure and binding sites of antigen, protein A, anti-p35 antibody, anti-p70 antibody and anti-p40 antibody. Adapted from Sommavilla et al, Protein Engin. Des. Sel. 2010*

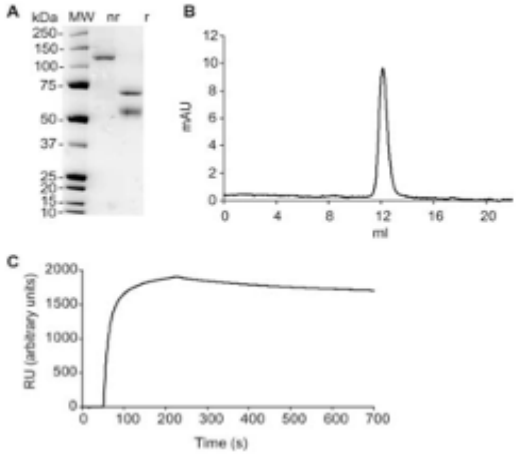

Figure 15 *Characterization of F8-IL12. (A) SDS – PAGE analysis of the protein under non-reducing and reducing conditions. (B) Gel filtration analysis reveals one single peak when analyzed under native conditions for the heterodimer F8-IL12. (C) Surface Plasmon resonance analysis shows flat dissociation phase of F8-IL12 (Biacore 3000, flow 20 ml/min).*

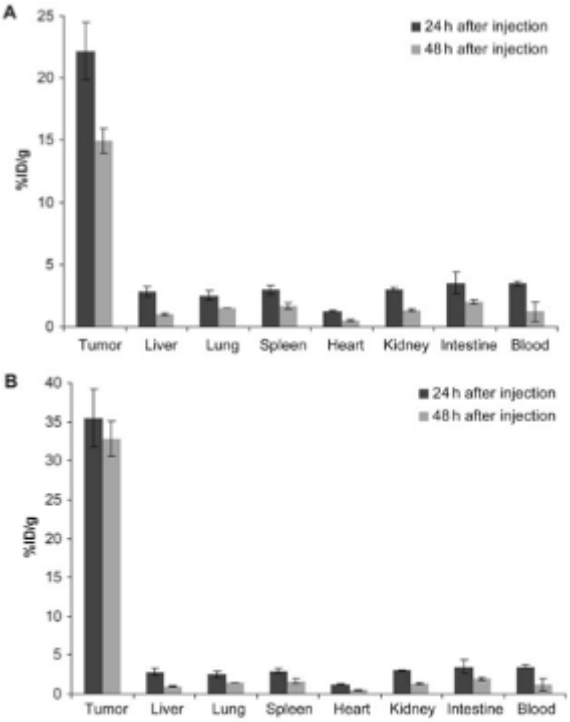

Figure 16 *Biodistribution studies. (A) F9 tumor bearing mice were injected intravenously with 125I-labeled F8-IL12 and sacrificed 24 or 48 h after injection. (B) Tumor values are corrected for tumor growth during the experiment (F9 tumors have a doubling time of 40 h).*

CLINICAL SIGNIFICANCE

The clinical significance of research in the field of vascular tumor-targeting immunocytokines is reinforced by the clinical results recently observed by the Philogen group, who has brought three immunocytokines (L19-IL2, L19-TNF, F16-IL2) to Phase II clinical trials in patients with various cancer types. The most advanced of these products is L19-IL2, which has exhibited numerous objective responses in a Phase IIa study with 32 patients and which is currently being investigated in a controlled Phase IIb in 90 patients with metastatic melanoma, in combination with dacarbazine.
Even though these clinical activities were not part of the ADAMANT Project, the positive results observed so far provide a strong rationale for research in the immunocytokine field.

Optimization of combination therapy with antibody conjugates (immunocytokines)

MILESTONES

- Preclinical antitumor activity of combination treatments for cytokine conjugates
- Antimetastatic activity of immunoconjugates in combination with chemotherapy in one preclinical model

OVERVIEW

The development of antibody derivatives in clinical trials relies on their use in combination with other anticancer treatments. In the frame of the ADAMANT project members of the Consortium have investigated the immunoconjugates described above (i.e. F16-IL2, F8-IL2, F9-IL2) in combination with standard-of-care chemotherapy or targeted drugs. A great effort to optimize the best treatment conditions on ad hoc mouse tumor models reflecting the tumor patient's characteristic was made.
Representative treatment regimens, described below, have yield cures in tumor bearing mice and represent promising candidates for clinical development.

F16-IL2 immunocytochine in combination with temozolamide on glioblastoma

Temozolamide - the standard of care for newly diagnosed glioblastoma patients - in combination with F16-IL2 - a clinical-stage immunocytokine consisting of human interleukin (IL)-2 fused to the human antibody F16, specific to the A1 domain of tenascin-C- was investigated on a model of glioblastoma . Immunohistochemical analysis with human glioblastoma surgical specimens and with U87 xenografts showed a strong and selective tumor staining using the F16 antibody. A quantitative biodistribution in nude mice confirmed the preferential accumulation of the radiolabeled F16-IL2 at the tumor site. In the therapy study, the combination of F16-IL2 with temozolomide induced a complete tumor remission in the animals, (**Figure 17** top panel). The same treatment led to a substantial size reduction of the U87 tumors growing iorthotopically in the the brain (**Figure 17** bottom panel), compared to temozolomide or the immunocytokine administered alone. The combined use of temozolomide and F16-IL2 may deserves clinical investigations, which will be facilitated by the excellent safety profile in cynomolgus monkeys, and by the fact that F16-IL2 is currently being investigated in clinical trials in patients with metastatic cancer [Pedretti et al, Br J Cancer 2010].

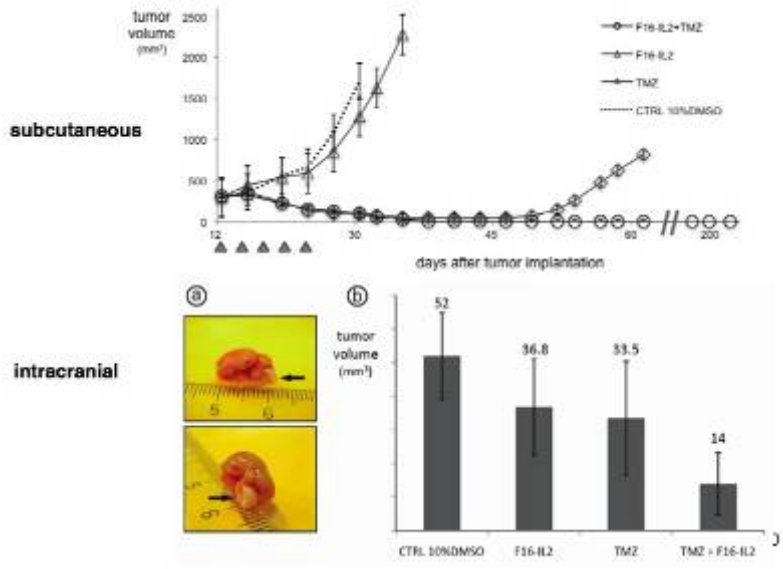

Figure 17 *F16.IL2 in combination with temozolamide on glioblastoma model (adapted from Pedretti et al, Br J Cancer, 2010)*

F8-IL2-immunocytokine in Combination with taxanes on melanoma model.

Human melanoma is characterized by strong vascular and stromal positivity for the EDA domain of fibronectin (EDA-Fn) and the pattern of EDA-Fn expression in melanoma xenograft models fully replicated that of the patient lesion. We found that F8 –an antibody directed against EDA fibronectin- conjugated to interleukin 2 (F8-IL2) and combined with paclitaxel (PTX), but not decarbazine (DTIC), completely inhibited the growth of human melanoma xenografts transplanted intradermally, with 80% complete regressions (**Figure 18** B). Furthermore we show the particular role of paclitaxel in this combination regimen. Infact a) PTX increased tumor vessel perfusion and permeability in melanoma xenografted tumors (see DCE-MRI, **Figure 18** A), favoring the uptake of F8 antibody and b) PTX prones the infiltration of NK cells and macrophages in melanoma xenograft. **Figure 18** C shows that the same regimen of paclitaxel combined with F8-IL2 immunocytochine inhibited the metastasis formation to the lung of treated mice. These preclinical studies, showing complete tumor regressions and antimetastatic activity, endorse the use of chemo-immunotherapy in the treatment of malignant melanoma and suggest that PTX is endowed with properties affecting the tumor stroma that deserve attention for the design of treatment regimens. [Moschetta 2011 (submitted)].

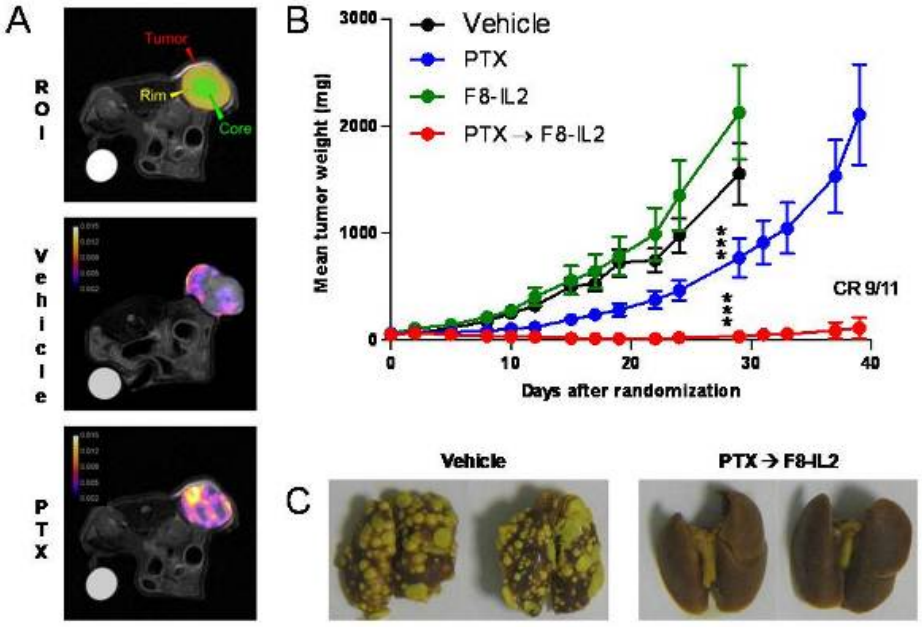

Figure 18 *F8-IL2 and paclitaxel (PTX) on melanoma xenograft model.*
A*) DCE-MRI showing that PTX promoted tumor perfusion in tumor growing intradermally.*
B*) Therapeutic activity of F8-IL2 immunocytokine in combination with PTX in human melanoma xenograft expressing EDA-Fn.*
C*) F8-IL2 immunocytokine in combination with PTX significantly reduces tumor burden in the lungs of mice. (adapted from Moschetta et al, 2010 -submitted)*

L19-IL2 immunocytokine in combination with rituximab on non-Hodgkin lymphoma (NHL)
The antibody-mediated delivery of therapeutic agents was also investigated in hematologic malignancies. The EDB domain of fibronectin, is expressed in B-cell non-Hodgkin lymphoma (NHL) and the human monoclonal anti-EDB antibody L19 can selectively localize to the lymphoma-associated subendothelial extracellular matrix. *In vivo*, the preferential accumulation of the antibody at the tumor site was confirmed by quantitative biodistribution analyses with radioiodinated antibody preparations. The fusion protein L19-IL2, which mediates the delivery of interleukin-2 to the neovasculature, displayed a superior antilymphoma activity compared with unconjugated IL2 in localized and systemic xenograft models of NHL. When coadministered with rituximab, L19-IL2 induced complete remissions of established localized lymphomas and provided long-lasting protection from disseminated lymphoma. The combined use of rituximab and L19-IL2, which dramatically increases the infiltration of immune effector cells in lymphomas, may deserve clinical investigations, facilitated by the fact that L19-IL2 is currently being studied in phase II clinical trials in patients with solid tumors [Schliemann et al, Blood 2009].

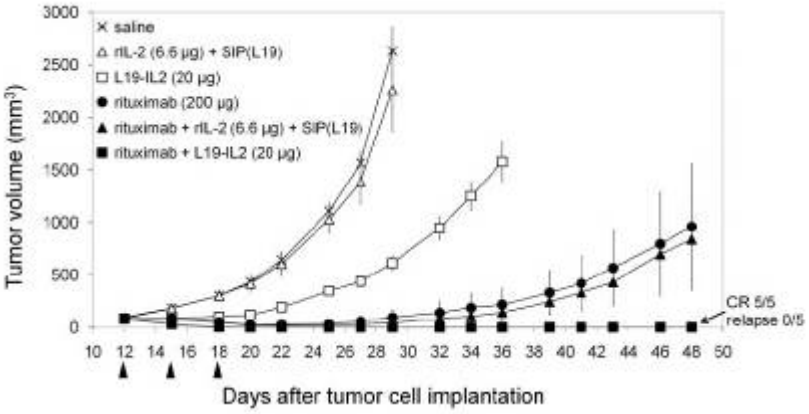

Figure 19 *Therapy studies with L19-IL2, used alone or in combination with rituximab in a mouse model of human lymphoma. (adapted from Schliemann et al, Blood 2009)*

PET clinical validation (microdosing)

MILESTONES

- The clinical evaluation of a promising anti-vascular antibody in an immuno-PET microdose phase 0 trial.

OVERVIEW
Immuno-PET has the potential to become a very powerful imaging tool for efficient clinical antibody development, especially because this technique allows accurate quantification. With relatively few patients the antibody can be characterized for e.g. the following key features:
1) tumor targeting capacity, and homogenicity of tumor targeting,
2) possible cross-reactivity with normal tissues,
3) residence time in blood, tumor and normal tissues,
4) possible complex formation in blood,
5) catabolic routing, and
6) inter-patient variation.

Microdosing (phase 0) studies as described by the European Agency for the Evaluation of Medicinal Products (EMEA), could be used to compare upfront compounds with similar intended Mode of Action but potentially different PK/PD characteristics, thus weeding out unsuitable agents for clinical development PRIOR to extensive clinical investigations in patients. These studies do not need the full set of toxicity and other safety studies. We anticipated, that also for antibodies the "microdosing" principles will be applicable, especially when there is good evidence that MAb biodistribution will not be strongly dependent on MAb dose.
The aim of was the clinical evaluation of a promising anti-vascular antibody in an immuno-PET microdose phase 0 trial.

^{124}I-F16SIP: aphase 0 single microdose study

Based on available preclinical data F16SIP, directed against the alternatively-spliced domain A1 of tenascin, was selected for phase 0 studies in operable head and neck cancer patients. All consortium partners were involved in the design of the study. The following EMEA-released guidelines and position papers were considered: "Note for guidance on the pre-clinical evaluation of anticancer medicinal products", "Note for guidance on the pre-clinical evaluation of biotechnology-derived products", "Note for guidance on non-clinical safety studies for the conduct of human clinical trials for pharmaceuticals", and "Position paper on non-clinical safety studies to support clinical trials with a single microdose". Since the clinical trial was planned to be performed at VU University Medical Center in the Netherlands, discussions were performed with Dutch regulatory authorities about the non-clinical safety studies needed to allow a clinical immuno-PET study with a single low dose of antibody. It became clear that 30 nMol MAb is allowed for phase 0 clinical trials, and that toxicity studies performed with cold F16SIP are also valid for studies with radiolabeled F16SIP, as long as the binding and pharmacokinetic properties of the MAb are not altered by the labeling procedure.

Based on this information the following steps were made:

i. GMP produced clinical-grade batch of F16SIP was made available with the consortium.
ii. Non-clinical safety studies were performed according to aforementioned regulation.
iii. A tissue cross reactivity test was performed according to FDA recommendations.
iv. Procedures and a manufacturing infrastructure for GMP production of ^{124}I-F16SIP were set up. An Investigation Medicinal Product Dossier (IMPD) of ^{124}I-F16SIP was written
v. A clinical protocol entitled: "A phase 0 single microdose study to evaluate the pharmacokinetics/-dynamics and specific tumor targeting of 124I-F16SIP in head and neck cancer patients", was developed and approved by METC
vi. Three head and neck cancer patients were enrolled and treated in this trial and two other patients are planned.

The first patient had a T2N0M0 lateral tongue carcinoma on the left side. The first PET-CT scan at 30 min after injection showed mainly blood pool. The second PET-CT scan at 24 h after injection showed clear uptake of ^{124}I-F16SIP in the left lateral tonque, corresponding to the localization of the primary tumor (**Figure 20**). During surgery at 168 h after injection samples were taken from blood, tumor, skin, healthy mucosa, fatty tissue and muscle. For assessing uptake of ^{124}I-F16SIP, expressed as the percentage of injected dose per gram tissue. Highest uptake was found in tumor tissue with a tumor-to-blood ratio of 8.2.
The second patient had a T2N0M0 cheeck carcinoma. The second PET-CT scan at 24 h after injection showed some uptake of ^{124}I-F16SIP in the tumor area, while biopsies at 120 h after injection revealed a tumor-to-blood ratio of 6.6.
Because of slow patient accrual, The original study protocol was amended in a way to limit the number of PET scans to 2 in order to avoid that patients have to travel to the clinic on several days in a row. In the mean time a third patient has been included in this phase 0 trial, and the trial is expected to be finished within a couple of months.

Results from this phase 0 trial thusfar show that F16SIP is a promising antibody for targeting antigen-positive tumors like head and neck tumors. As such, this antibody is candidate to be used for tumor detection, and when coupled to a toxic payload for selective tumor eradication.

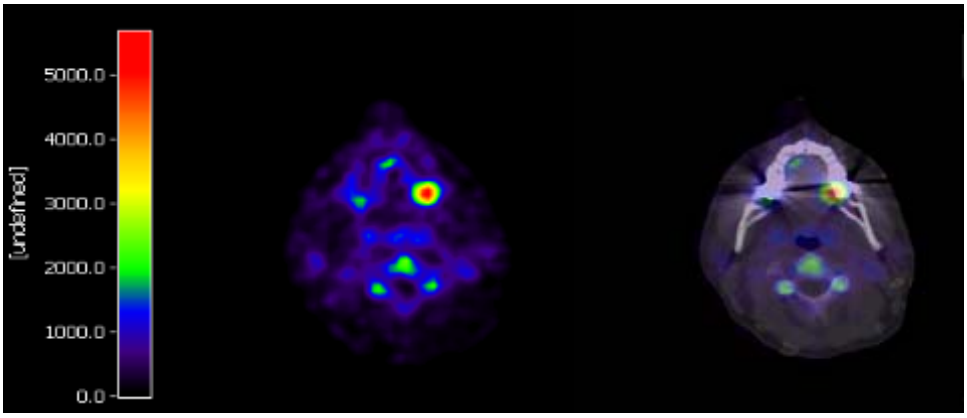

Figure 20 *PET-CT image with ^{124}I-F16SIP in head and neck cancer patient showing selective targeting of tumor in lateral tongue*

Regulatory affairs and strategic monitoring

MILESTONES

- Design of Clinical Development Plan and protocol for Phase 1 FIM (first-in-man) study
- Micro-Dosing Study protocol
- Organization of a multidisciplinary oriented focus group (FG)

OVERVIEW

The mission of ADAMANT was to discover new potentially "druggable" anticancer targets in solid tumors and generate higly effective new monoclonal antibodies against these targets minimizing collateral damage to the normal tissues by homing the delivery of toxic agents (chemical o natural products molecules, radionuclides or immunostimulatory cytokines) specifically to the cancer cells or the cells of the cancer-supporting scaffold (stroma)
to the cancer cells or the cells of the cancer-supporting scaffold (stroma)

The challenge of the project was to identify these new potentially "druggable" targets in a preclinical framework mimicking as much as possible the ultimate setting in which these antibodies would be used (i.e. the cancer patient). Indeed since the inception, it was recognized that the traditional "bench to bedside" framework could be converted to a more innovative "bed to bench-side" approach retrofitting the constructs of the preclinical experiments from the actual clinical settings,

With this challenge in mind a task was devised to support the project and indeed relayed mainly on the collaboration with the more "clinical oriented" tasks: optimization of the combined therapy with other agents; PET clinical validation and RIT.

SENDO (Southern European New Drug Organization) was involved in the Project to provide, in connection with the performance of the preclinical activities, the expertise in clinical drug development and specifically in preparing dossiers, designing pharmacodynamic and pharmacokinetics based phase Zero and Phase I trials, and running them in cancer patients at selected and qualified clinical centers.

The organizational model of SENDO is that of an Academia/Industry go-between geared at understanding the needs and fulfilling the missions of both stake-holders. During the project SENDO promoted and supported innovative anticancer drug research stimulating interaction with industry in the early stages of drug development. Working trough its SME, SENDO TECH, the Foundation is committed to guarantee the highest level of quality research, validity and reliability of clinical data collected, having in place established Standard Operating Procedures (SOPs) for data collection, logistical organization of the trial, monitoring activities and medical writing. SENDO TECH is a certified CRO in Italy and France since 2009 and 2010 respectively.

Within ADAMANT the full quality-controlled radiological assessment of clinical response in the patients included in the phase I first in man studies (including the "blinded" revision of all the radiological assements of patients treated with L19-IL2) was provided; in addition the protocol templates for phase 0 and phase I FIM studies, and all kinds of quality controlled study report templates were designed and finalized. Based on that, the study report of the first FIM already concluded was produced and clinical data have been presented at two International Conferences during 2010: ASCO [Del Conte et al, JCO (May20 suppl) 2010] and ASTRO [Chiesa et al, Int J Rad Oncology Biology Physics (suppl) 2010].

Regulatory assistance to define controversial ethical and legal aspects of phase 0 clinical trials in Europe was provided. Within this framework the first PET phase Zero IMPD (Investigational Medicinal Product Dossier) was finalized and a submission for a microdosing study was done by VUA. An ImmunoPET clinical study has been designed and activated .

Finally to facilitate the strategic monitoring of the results, multidisciplinary oriented focus groups have been organized to discuss and analyze the open questions on PET in translational oncology and on pharmacodelivery of cleavable linkers drugs. The discussions helped the creation of "platform of concepts " stemming from the project collective experiences and specific expertises. This platform could become both a "conceptual signature" of the ADAMANT project and/or a springboard for future scientific explorations of the ADAMANT participants. Some of the Focus debated issues have been subjects of pubblications [Frey et al, Integr Biol (Camb) 2011 ; Roesli and Neri, J Proteomics 2010; Moschetta et al, Curr Pharm Des. 2010]

Exploitation of the experimental results, through suitable patent applications and industrial feasibility studies, and the dissemination of accomplishment and technologies.

To assure the visibility of the Adamant results the Consortium has disseminated information through various channels involving different targets. There are communications by the coordinator such as the website addressing the general public and two ads appearing in the magazine "The Parliament" addressing European decision makers, and other dissemination activities that are done by individual participants mainly publications, congress presentations, talks addressing the scientific community.
The project website has been available since the start of the project at the address www.adamant-fp7.eu and has been periodically updated, providing news of major events of the Consortium and the end of the project. The website will be maintained online after the end of the project.

All participating groups have published their research results either alone or together with other beneficiaries in peer-reviewed scientific journals. A total of 50 papers in peer reviewed journals have been published (of which 14 open access), and more than 70 congress presentations given. More manuscripts (n=14) are under preparation and submitted or accepted for publication. In several instances beneficiaries scientific representatives have been invited as speakers at national and international meetings.These activities will continue as expected.

A workshop on Tumor Microenvironment and metatases was organized by the Belgian Society for Cell and Developmental Biology in Liege, March 27, 2010. in collaboration with three FP7 projects, ADAMANT, MicroEnviMet and Tumic.

Personal training programs were facilitated among the partners of the Adamant consortium.
For example, Fabia Doll, Master Student at ETH Zurich (B2), performed her master thesis at UCL (B7) from May to July 2008.
Dr. Andrei Turtoi, scientist at ULG (B8), spent a 4-month training period (from June to September 2009) at UCL (B7) in the frame of his postdoctoral training to learn the technique of orthotopic liver metastasis in murine models grafting colorectal tumor cells into the liver via splenic injection.
Antonietta Silini performed her PhD thesis in cooperation between IRFMN (B1) and UCL (B7) and spent 1 month (July-August 2009) at UCL.
Dr. Sreejith P Shankar, postgraduate student at KemoTech (B4), spent a 3-months secondment period from Feb to Apr 2009 at VUA (B10) during his PhD training.
Francesca Pretto is a PhD student of ETH Zurich (B2), from Jan 2010 she is performing part of her research at IRFMN (B1)

Twenty doctoral, PhD and graduation theses have been promoted by the Beneficiaries on subjects connected with the ADAMANT project, 11 have been completed, 9 are still in progress.

A total of 6 patent applications were filed with EPO and two more are under evaluation.

On occasion of major meetings of the Consortium, on three occasions a Research Valorization Roundtable was held with the experienced support of the Advisory Board. Aim of this Roundtable was to assure the assessment of the societal and industrial exploitation of results and best return on investment of the project. Progress presented by the Adamant consortium was analyzed in detail by the members of the Advisory Board, resulting in suggestions from people not directly involved in the project. Analyses were discussed in plenum and the advice allowed a focussed continuation of the work until the next major meeting.

4.1.4 The potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results

In spite of their commercial success, conventional monoclonal antibodies which are currently used for the treatment of solid tumors offer only an incremental benefit to cancer patients. For this reason, there is an urgent need for superior anti-cancer biopharmaceuticals. Not surprisingly, virtually all large pharmaceutical companies are heavily investing into biomedical strategies for the arming of monoclonal antibodies with bioactive payloads [Webb et al, Nature Biotechnol. 2011].

The potential impact of the ADAMANT Project can be analyzed in terms of four main milestones achieved over the last three years:

i. the discovery and validation of novel markers of tumor neo-vasculature and stroma, followed by the generation and characteriation of human monoclonal antibodies
ii. the development and implementation of novel antibody-based therapeutic strategies
iii. the stimulation of innovative clinical trials for the treatment of patients with incurable forms of cancer
iv. the creation of new jobs, in terms of formation of new companies and/or growth of existing companies

Traditionally, markers of tumor angiogenesis and of the tumor stroma have been discovered by serendipity. Out of many hundred different antibodies which are routinely analyzed by immunohistochemistry, it would occasionally happen that the study of a new monoclonal antibody would reveal a staining pattern coinciding with tumor neo-vascular structures. Markers of angiogenesis, such as the EDB domain of fibronectin, endoglin, PSMA, have all been discovered this way. The ADAMANT Project has contributed to the development and implementation of methods for the systematic characterization of vascular proteins in health and disease. We now have Atlases of vascular markers for various tumor types, which allows for the first time to choose a target for antibody product development based on an unbiased comparative evaluation of expression patterns. We are confident that the quality of the tumor-associated antigen used for product development will ultimately determine the quality of the cognate antibody-based product.

On the second aspect of ADAMANT's potential impact (novel antibody strategies), it must be mentioned that it is still not clear what will be the most successful strategy for arming antibodies. Different strategies have distinctive favorable features and, at the same time, carry distinctive liabilities. For example, radiolabeled antibodies may be ideally suited for the therapy of radiosensitive tumors (e.g., lymphomas), but are less effective for radioresistant tumors or for tumors which do not exhibit an adequate antibody uptake compared to normal tissues. For this reason, the ADAMANT Project has chosen to focus on three main antibody functionalization strategies (radiolabeling, fusion to cytokines, coupling to drugs with cleavable linkers) and has made an impact in all these areas. Indeed, the Philogen group (one of the partners in the Consortium) has brought seven armed vascular targeting antibodies to Phase I and to Phase II clinical trials.

At present, the most promising clinical results with armed vascular targeting antibodies have been reported for the (i) radioimmunotherapy of patients with incurable forms of lymphomas; and (ii) therapy of metastatic melanoma patients with IL2-based or TNF-based immunocytokines.
In the first case, multiple major responses have been observed in patients with Hodgkin's lymphoma, who had failed all other therapeutic options [Sauer et al, Blood 2009].

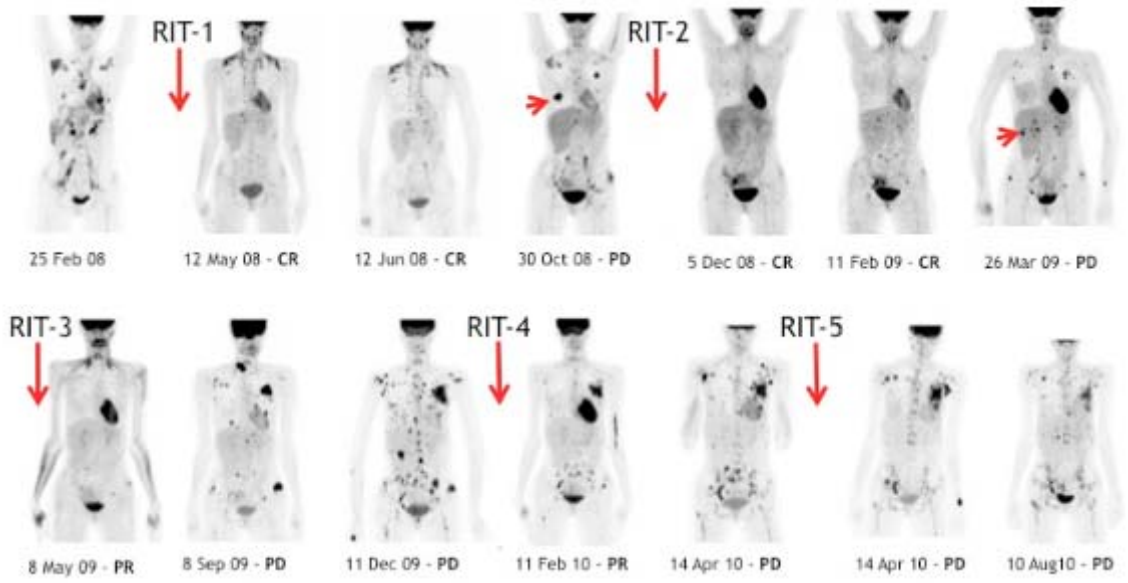

Figure 21 *Repeated responses in a patient with Hodgkin lymphoma monitored by FDG-PET, as a result of treatment with the L19 antibody, labeled with iodine-131*

In the case of IL2-based immunocytokines, the treatment of metastatic melanoma patients with L19-IL2 plus dacarbazine has led to the doubling of median overall survival compared to historical control patients, treated with dacarbazine alone [Eigentler et al, data presented at ASCO 2011 and manuscript submitted]. This treatment modality is currently being investigated in Phase IIb clinical trials in Germany, Italy and Austria. Importantly, the recent discovery that taxanes may be better suited for the combined use with IL2-based immunocytokines and that curative effects can be observed when paclitaxel is administered before the immunocytokine (not after) will stimulate the execution of novel combination trials for the treatment of patients with metastatic melanoma.

The implementation of the first Phase 0 immuno-PET clinical trial in Europe (i.e., the use of the F16 antibody labeled with iodine-124 for the imaging of cancer patients) is another important milestone of the Project. We expect that in the future targeted antibody drugs will be developed in combination with imaging modalities, which may guide patients selection and may reveal a correlation between responding patients and patients with a good antibody uptake in the tumor.

The companies which have participated in the Project have grown substantially during the activities of the ADAMANT Project. PHC has opened new laboratories in Otelfingen and has created new jobs [Philochem AG (and its mother company, Philogen) now have approximately 80 employees, between the Siena and Otelfingen site]. KTECH has made substantial contributions to the development of tubulysin derivatives as an innovative and general class of cytotoxic agents for the coupling to antibodies. Moreover, Kemothech srl has recruited two employees thanks to the results achieved. Finally, Targetome is a spin-off company of the University of Liege, which has been founded as a result of innovative technologies in target discovery and validation developed in the frame of the ADAMANT Project.

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