UPTEC X 11 023
Examensarbete 30 hp
April 2011

Functional characterisation *in vitro* of an Affibody® molecule aimed for vascular targeted tumor therapy

Erika von Hage

UPPSALA
UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 11 023 | **Date of issue 2011-04**

Author

Erika von Hage

Title (English)

Functional characterisation *in vitro* of an Affibody® molecule aimed for vascular targeted tumour therapy

Title (Swedish)

Abstract

In a certain type of cancer immunotherapy, IL-2 is administered in high doses to the patient in order to trigger an immune response against tumour cells. The high dose administered leads to high general toxicity. By targeting IL-2 to the sites of the tumours, the dose can be decreased and toxicity reduced. Here, a PDGFR-β specific fusion protein (Z03358-ABD-IL2) aimed for delivery of IL-2 to tumour vasculature, has been characterised *in vitro* for IL-2-activity and target binding. Also, two PDGFR-β specific Affibody molecules (Z02465 and Z02483) have been characterised *in vitro* for target binding and biological blocking activity. Results showed that Z03358-ABD-IL2 had full IL-2 activity, but decreased target binding. In contrast, full target binding and blocking capacity was confirmed with Z02465 and Z02483. From this work it can be concluded that Z03358-ABD-IL2 has activity in all parts, but re-cloning with Z02465 or Z02483 should be considered for full target binding capacity.

Keywords

Cancer, tumour vasculature, tumour targeting, IL-2, Affibody molecule, pericyte, PDGFR-β

Supervisors

Fredrik Frejd & Ingmarie Höidén-Guthenberg
Affibody AB

Scientific reviewer

Angelica Loskog
Uppsala universitet

Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **36**

Biology Education Centre Biomedical Center Husargatan 3 Uppsala
Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 471 4687

Dedicated to mom that died in kidney cancer spring 2008

Functional characterisation *in vitro* of an Affibody® molecule aimed for vascular targeted tumour therapy

Erika von Hage

Populärvetenskaplig sammanfattning

Cancer är en av vår tids största folksjukdomar och var tredje person kommer att drabbas någon gång under sin livstid. Lyckligtvis går forskningen framåt och nya cancermediciner och behandlingssätt utvecklas kontinuerligt. Eftersom cancer egentligen är ett samlingsnamn på ca 200 sjukdomar med okontrollerbar celltillväxt som gemensam nämnare, finns det många olika behandlingssätt. I en viss typ av immunoterapibehandling, ger man interleukin-2 (IL-2) i höga doser till patienten i syfte att få igång kroppens eget immunförsvar till attack av tumören. Den höga dosen IL-2 ger en hög toxicitet i kroppen som medför svåra biverkningar. Genom att istället specifikt målsöka tumörvävnaden med IL-2 kan dosen av läkemedel minskas avsevärt. En strategi att göra detta på är att målsöka IL-2 till blodkärl vid tumören. När IL-2 ansamlats i hög dos lokalt i tumören, aktiveras bland annat NK-celler och cytotoxiska T-celler att gå till attack mot tumörvävnaden och förstöra den.

Detta examensarbete har varit en del i ett större projekt med syfte att ta fram en ny läkemedelskandidat baserad på en tumörvaskulatur-målsökande Affibody-molekyl bunden till IL-2. Den konstruerade molekylen var gjord för att målsöka tyrosinkinas receptorn platelet-derived growth factor β (PDGFR-β) som uttrycks av så kallade pericyter i tumörvaskulaturen. Molekylen bestod av en PDGFR-β specifik Affibody-molekyl (Z03358) fuserad med en albumin bindade domän (ABD) och IL-2. Examensarbetet har också innefattat två PDGFR-β-specifika Affibody-molekyler som inte var fuserade med IL-2 (Z02465 och Z02483). Alla kandidater har i detta examensarbete testats för cellbindande funktion. IL-2 konstruktet har även testats för immunförsvarsstimulerande funktion och de två ofuserade Affibody-molekylerna har testats för ligandblockerande funktion av PDGFR-β. Resultaten visar att Z03358-ABD-IL2 har förmåga att aktivera IL-2 beroende cytotoxiska T-celler, men för att öka konstruktets cellbindande funktion borde en omkloning med Affibody-molekyl Z02465 eller Z02483 övervägas.

Examensarbete 30hp
Civilingenjörsprogrammet Molekylär bioteknik
Uppsala universitet april 2011

Table of contents

Concepts and abbreviations

ABD	Albumin Binding Domain
Angiopoietin	(-1 and -2) - Ligands of the Tie-2 receptor
CTLL-2	A cytotoxic T-cell line that is dependent of IL-2 for growth
ECM	Extracellular matrix
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HER-2	Human Epidermal Growth Factor Receptor 2
HSA	Human Serum Albumin
IFN-γ	Interferon γ
IL-2	Interleukin 2
NIH-3T3	Mouse embryonic fibroblast cell line
NK-cell	Natural Killer cell
PDGF-BB	Platelet Derived Growth Factor BB
PDGF-DD	Platelet Derived Growth Factor DD
PAE-cells	Porcine Aortic Endothelial cell line transfected with PDGFR-β
PDGFR-β	Platelet Derived Growth Factor Receptor β
α-SMA	α-Smooth Muscle Actin
SPA	Staphylococcal Protein A
Tie-2	Receptor tyrosine kinase expressed on vascular endothelium
TGF-β1	Transforming Growth Factor β1
VEGF-A	Vascular Endothelial Growth Factor A
VEGFR-2	Vascular Endothelial Growth Factor Receptor 2

1. Introduction

1.1 The tumour and its environment

Cancer is characterised by an uncontrolled cell growth and cells that do not respond to apoptosis signals. In healthy tissue, normal cells follow a preprogrammed schedule of growth, division and eventually cell death (apoptosis) but when this process is impaired due to genetic alterations, cancer can begin to form[1]. Tumour cells have a migratory phenotype. They induce remodeling of the surrounding extracellular matrix (ECM) in order to invade surrounding healthy tissue and metastasize through lymphatic or blood vessels. Tumour cells can also modify normal cells to produce chemokines, growth factors and matrix-degrading enzymes, all enhancing proliferation and invasion of the tumour. The cancer progression does not only involve the cancer cells. The microenvironment in a tumour promotes cancer initiation and growth. Supporting structures in the microenvironment includes lymphatic vascular networks, endothelial cells, pericytes, stromal fibroblasts, infiltrating immune cells, and the extracellular matrix[2]. In order for the tumour to grow, vasculature is recruited to secure its own blood supply to deliver nutrients and oxygen. The tumour vasculature is formed both by angiogenesis, a process where new vessels grow from pre-existing vessels into the tumour and by vasculogenesis, that involves the recruitment of endothelial progenitor cells [2, 3]. The angiogenesis in tumour tissue, contrary to normal tissue, results in a poorly organized vasculature with chaotic, tortuous and leaky vessels unable to support efficient blood flow. This chaotic tumour vasculature is a result from an imbalanced expression pattern of several angiogenic factors[2]. Vascular maturation and angiogenesis involves interactions between pericytes and endothelial cells, two types of cells important for blood vessel structure[2, 4]. Interactions between these cells involve growth factor signaling pathways with the VEGF-A/VEGFR-2, PDGF-BB/PDGFR-β, TGF-β1, Angiopoietin/Tie-2 systems[3].

1.2 Pericytes

Blood vessels are mainly composed of two interacting cell types. Endothelial cells, forming the inner wall of the blood vessel, and perivascular cells, wrapping the outside of the blood vessel[3, 4]. For a long time, cancer research on therapeutic intervention by targeting blood vessels has mainly focused on the endothelial cell. However, quite recently, pericytes (a type of perivascular cell) have gained a renewed scientific interest as a target for anti-angiogenic therapy due to its important regulatory functions in tumour angiogenesis [3, 4]. The pericyte is physically supporting endothelial cells and the two cells interact by exchanging ions and small molecules through small gap junctions at the surface. The pericyte is a crucial regulator of the endothelial cell function, as well as for the formation of the vasculature. Loss of pericytes causes leaking vessels, which lead to conditions such as for example edema[4]. Pericytes also function in the control of recruitment and regulation of tumour fibroblasts. Molecular markers of the pericyte are: α-smooth muscle actin (α-SMA), nonmuscle myosin, desmin, tropomyosin, high

molecular weight melanoma associated antigen (NG2), aminopeptidase A and N, nestin, sulfatide and PDGFR-β. The expression level of these markers varies with the type of vessel and marker expression can also vary in different organs and under pathological conditions such as cancer[5]. It is well documented that the endothelial cells in the tumour vessel differ from endothelial cells in normal vessels, but it has been less clear whether pericytes are abnormal in tumour vessels. However, results in mice suggest that pericytes in tumour vessels differ from normal vessels. For example, studies have shown that pericytes in tumour vessels have an altered expression of marker proteins. The altered marker expression during pathological conditions appears when the tumour grows and could be a problem when identifying the pericytes with one single marker. Unfortunately not much is known about the marker expression variability since most studies uses only one marker, usually PDGFR-β [5]. Pericytes in tumour vessels are dynamic and can remodel existing vessels and induce formation of new vessels by angiogenesis. Structurally, tumour pericytes seem to be more loosely attached to the vasculature with extended cytoplasmic processes deep down in the tumour parenchyma. Pericytes in some tumour tissues also seem to be less abundant than pericytes in normal tissue. However, studies indicate that only small amounts of pericytes are needed in order to carry out their important functions [4, 5]. Despite the rather abnormal structure of the tumour pericyte, it does provide functions that are necessary for regulation of pathological angiogenic processes, endothelial cell survival and vessel maintenance[2, 4]. However, abnormalities of the pericytes in tumour vessels may be beneficial when targeting these vessels since the normal vasculature has an increased chance of escaping damage[5].

1.3 Platelet-derived growth factor β (PDGFR-β)

Platelet-derived growth factor β (PDGFR-β) is expressed on the surface of connective tissue cells such as smooth muscle cells, pericytes, fibroblasts, macrophages and some tumour cells. The receptor binds two ligands: PDGF-BB and PDGF-DD. Binding of PDGF-BB to the receptor leads to proliferation and migration of the PDGFR-β-expressing cell. The expression of the receptor on pericytes is a common characteristic of solid tumours and various studies have shown that both PDGFR-β and the ligand PDGF-BB is expressed and upregulated in most solid tumours[6, 7]. The expression of the upregulated PDGFR-β receptor in tumours, results in an abnormal and excessive angiogenesis that nourishes the tumour and stimulates its growth. PDGF-BB can stimulate the growth of certain tumour cells in both a paracrine and an autocrine way and PDGF-BB, produced by cancer cells, can act on non tumour cells, indirectly leading to growth of the tumour, even though cancer cells are not responding to the ligand themselves[6].

1.4 Targeting of the vasculature in the tumour microenvironment

The components of the tumour microenvironment such as pericytes, endothelial cells, and carcinoma-associated fibroblasts (CAF:s) are interesting non-tumour cell targets[7]. Targeting the microenvironment can provide advantages since non-tumour cells

presumably are more genetically stable than tumour cells. In contrast, tumour cells are genetically unstable and can acquire drug resistance and accumulate adaptive mutations[2]. Many anti-angiogenic agents have been developed and tested in preclinical experiments with good results. One of those anti-angiogenic therapies involves combined targeting of VEGFR-2, a marker for endothelial cells, and PDGFR-β, a marker for tumour pericytes. Results show that blood vessels regress and tumour growth is effectively reduced in treated mice[9, 10]. Further on, when targeting tumour vasculature with the inhibitors binding VEGFR-2 and PDGFR-β and then combine targeting with radiation tumour therapy, the effect of radiation therapy in mice is improved [11]. In another study, combination of chemotherapy and PDGFR-β targeting enhanced the effects of chemotherapy in mice [12]. Interestingly, when reducing pericyte activity by using PDGFR-β inhibitors as single agents, limited effects have been achieved[12]. Combinations of inhibitors that target pericytes and endothelial cells by blocking PDGF and VEGF signaling seem to work more effectively than any of the inhibitors individually. The same applies for the combination of PDGFR-β inhibition and chemotherapy or VEGFR-2/PDGFR-β inhibition and radiotherapy. Thus, combination strategies will likely prove most effective when using angiogenesis inhibitors[2, 11, 12, 13].

1.5 The Affibody molecule and ABD

The Affibody molecule is a small (7 kDa, 58 amino acids) robust protein that can be isolated for high affinity and specificity to most protein targets. It is based on the cystein-free, three helical bundle Z-domain which originates from the B-domain of the immunoglobulin-binding region of staphylococcal protein A (SPA) [14, 15, 16]. The Affibody molecule is thus not related to antibodies but it mimics the functionality[14, 16]. Structurally, the Affibody molecule is less complex and smaller than an antibody and this property offers advantages, both in applications and in production. The Z-domain was chosen for the construction of the Affibody molecule since it has many advantageous properties such as a high solubility, relatively high thermal stability, a kinetic of the folding reaction that is one of the fastest reported for a protein and since it could be produced with high yields in *Escherichia coli*. Combinatorial libraries of a wide range of Affibody molecules have been created by randomizing 13 amino acids in helices one and two of the Z-domain (see fig.1). From these libraries, high affinity Affibody molecules have been selected to a large range of targets including PDGFR-β[7, 14, 15, 16].

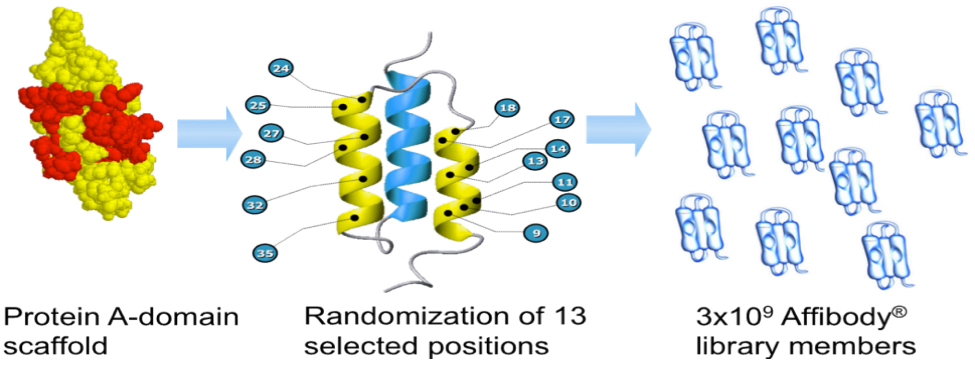

Figure 1. Randomization of 13 selected positions of protein A-domain scaffold in order to create combinatorial libraries of high-affinity Affibody molecules. Illustration used with permission from Affibody® AB.

In order to optimize the technology, a second generation Affibody molecule scaffold was established. Improvements were achieved by substituting 11 amino acids in the non-binding surface of the old scaffold, making the surface distinctly different from the parental scaffold. By these changes, an improved chemical and thermal stability, increased hydrophilicity and enhanced amenability for peptide synthesis in large scale was achieved, compared to the parental molecule. The goal was also to remodel the Affibody molecule in order to reduce similarity to the original B domain of Protein A and to reduce any residual interactions with immunoglobulins[16]. The second-generation Affibody molecule with reengineered scaffold was then used in targeting of HER2-expressing tumours and the molecule showed excellent targeting capacity in tumour-bearing mice, despite reengineering of the nonbinding surface[17].

The therapeutic efficacy of small protein drug candidates is limited by a short *in vivo* serum half-life. The main reason for their rapid clearance is due to the small size of the molecules, which is below the clearance threshold of the renal system. Another reason is the risk of degradation by serum and intracellular proteases[18]. In order to overcome the difficulties and improve the properties of the pharmacokinetics, a variety of strategies have been developed. One of the strategies is to associate the drug to a long-lived plasma protein such as albumin. Albumin has a long half-life (approximately 2-3 weeks in humans). It is present in human plasma at 50 mg/ml and is therefore one of the most abundant serum proteins in blood. By fusing the Affibody molecule with an albumin-binding domain (ABD) (see fig. 2), binding to the patient's own serum albumin with a high affinity is allowed. Thereby, the size of the albumin-fusion is above the renal clearance threshold and it adopts the long plasma half-life of serum albumin. By the extended half-life, a less frequent dosing of a biopharmaceutical may be achieved and peak concentrations in the blood are decreased which reduces the risk for adverse events. The fusion also has the wide and rapid biodistribution of serum albumin, thereby not only reducing the uptake in the kidneys, but also facilitating distribution in the interstitial compartment. There are different variants of ABD available. In this project, a monovalent variant of an albumin-binding motif, originating from the streptococcal protein G was used[18, 19].

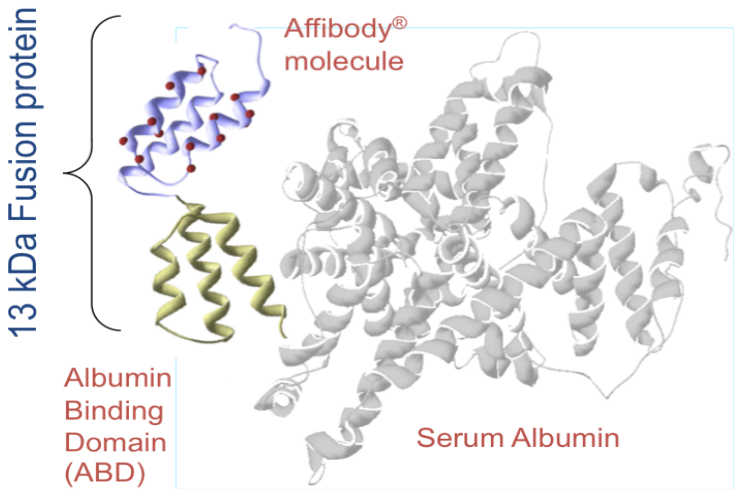

Figure 2. Affibody molecule fused to ABD. A serum albumin is shown for comparison. Illustration used with permission from Affibody® AB.

1.6 Tumour immunosuppression

It has long been recognized that the immune system plays a role in tumour cell clearance, but even though tumours do express tumour-specific antigens that could function as targets for T-cells, tumours evade the destruction by the immune system[21, 22]. Anti-tumour immune responses are either suppressed or not sufficiently vigorous in the tumour and both possibilities are supported by earlier studies [21]. Tumours have developed a wide range of properties in order to escape the immune system. These properties involve mechanisms such as mutations of the processing and presentation of antigen, secretion of immunosuppressive molecules by the tumour and resistance to apoptosis by over expression of anti-apoptotic molecules[20]. However, the immune system has the capacity of eradicating tumour cells by antitumour responses generated by for example cytotoxic T-cells and natural killer cells (NK-cells). By fusing a tumour target specific antibody to immunostimulatory interleukin-2 (IL-2), a high local dose of IL-2 may trigger the resting immune system to lysis of the tumour cells, a strategy that shows promising results in various studies[20, 23, 24].

1.7 Interleukin-2 (IL-2)

Interleukin-2 (IL-2) is a 15 kDa α-helical cytokine produced by antigen-activated T-cells, predominantly CD4+ and CD8+. IL-2 is required for both the induction and self-regulation of T-cell-mediated immune responses. IL-2 stimulates T-cell proliferation and initiates apoptic cell death of activated T-cells. It also stimulates the proliferation and effector functions of NK-cells and B-cells[25]. Since tumours are known to be immunosuppressive, IL-2 is today given to some cancer patients to activate the immune

system in order to detect and attack cancer cells. The problem is that large drug doses must be administered to the patient leading to unacceptable toxicities. By targeting IL-2 to the sites of the tumours, better anti-tumour efficacy and minimized side effects of IL-2 may be achieved. Studies show that targeting of IL-2 has lead to a dramatic enhancement of the therapeutic properties of the cytokine. In murine models, there are two mechanisms in which IL-2 can mediate antitumour effects. First, IL-2 treatments can activate preexisting antigen-specific T-cells to recognize and attack tumour tissue. The other way is by activating natural killer cells[26]. One example of the effects of targeted IL-2 is a study done 2002 when an immunocytokine consisting of a human vascular targeting antibody fragment (L19) fused to IL-2, selectively delivered IL-2 to tumour vasculature. The targeted IL-2 led to dramatically enhanced therapeutic properties of IL-2. Thus, tumours in L19-IL2 treated mice were significantly smaller and there was a notably higher accumulation of T-cells, NK-cells, macrophages and IFN-γ compared to tumours of control mice[27]. Further on, in a study from 2008, the combination of L19-IL2 and rituximab (an antibody specific for CD20 used in therapy of non-hodgkin lymphoma) increased the accumulation of immune effector cells in the tumour site, compared to L19-IL2 itself, and completely eradicated established lymphomas in tumour bearing mice[28]. L19-IL2 has been tested in clinical trials with good targeting properties, manageable toxicities and no treatment related deaths[29].

1.8 Aim of project

This degree project was part of an ongoing project with the aim to concentrate PDGFR-β targeting immunostimulatory molecules in tumour lesions in order to elicit therapeutic antitumour responses. The specific aim of the degree project was to characterise the construct Z03358-ABD-IL2 with respect to both IL-2 activity and target binding in the presence and absence of human serum albumin. Characterisation for target binding was done by flow cytometry and characterisation for IL-2 activity was done by a cell-assay using IL-2 dependent CTLL-cells. A comparison of the PDGFR-β binder in old (Z02465) and new (Z03358) scaffold was also done with flow cytometry and ELISA. All experiments were done in order to support future therapy experiments.

2. Materials and Methods

2.1 Cell culturing and treatment conditions

The cell lines used at Affibody AB in this project were PDGFR-β expressing mouse embryonic fibroblast cell line NIH-3T3 and murine T cell lymphoblast cell line CTLL-2 (CLS). NIH-3T3 cells were grown in DMEM (Gibco) supplemented with 10% fetal calf serum (Gibco), 1% L-glutamine (Lonza), 10% PEST (Lonza), 1% NeAA (Lonza), 1% Napyruvate (Lonza). CTLL cells were grown in RPMI (Gibco) medium supplemented with 10% fetal calf serum (Gibco), 1% L-glutamin (Lonza), 1% PEST (Lonza) and 10% rat T-Stim without conA (BD biosciences). All cells were cultured at 37°C in 10% CO_2 and split as recommended by the provider.

CTLL cells were harvested by transfer from culture bottle to a 50 ml falcon tube. After washing and counting, cells were diluted in assay medium appropriate to the experiment that was to be done.

NIH-3T3 cells were harvested according to protocol that follows. After removal of supernatant, the NIH-3T3 cells were washed twice using sterile PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 μm filtered). Trypsine-EDTA (Lonza) was warmed to 37°C and added to bottle, covering the bottom. The cells were incubated at 37°C for maximum 5 minutes, until all cells were detached from the bottle. Cells were separated by pipetting up and down a few times. Trypsination was stopped by adding some culture medium. After washing and counting, cells were diluted in assay medium appropriate to the experiment that was to be done.

Counting of cells was done in a Bürker chamber.

2.2 N-ethyl-maleimide conjugation (NEM)

In order to reduce cysteine bridges, DTT was added to Z03358-ABD-cys and Z03358-cys to a concentration of 20 mM. The samples were incubated on a shaker for 1.5 hours at 37°C. The buffer was exchanged to conjugation buffer (0.05 M NaH_2PO_4 + 150 mM NaCl) to remove excess DTT using PD10 desalting columns (GE Healthcare). In order to determine the amount of sample, the absorption at 280 nM was measured using a spectrophotometer (Nanodrop ND-1000). NEM conjugation of sample was done at 10x molar excess of N-ethyl-maleimid. Conjugation buffer was exchanged to final buffer (PBS) on PD10-columns to remove excess N-ethyl-maleimid. The samples were concentrated on a spin column (Amicon Ultra-15 Millipore, 3000NMWL). In order to determine the amount of sample, it was again analyzed by the spectrophotometer at 280 nM. The sample was finally analyzed by HPLC-MS analysis (Agilent Technologies, 1100 LC/MSD SL ESI System) to determine the degree of NEM-conjugation.

2.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Five µg of each Affibody sample was diluted in PBS to a volume of 12 µl. Four µl 4x LDS sample buffer (NP0007, Invitrogen) was added to the samples. Tubes were vortexed and heated to 70°C. After centrifugation for a few seconds, the samples and a sharp molecular weight standard (Novex® Sharp Pre-stained Protein Standard (LC5800 Invitrogen)) were loaded on a 15 well 4-12% NuPAGE gel (NP0322BOX, Invitrogen). The gel was run for 35 minutes at 200 V. After electrophoresis, the gel was stained in Coomassie blue on a shaker for one hour and destained in 30% ethanol for 30 minutes and 10% ethanol for one hour. Finally, the gel was washed and scanned.

2.4 Flow cytometry

NIH-3T3 cells were harvested, washed, counted and resuspended in culture medium to an approximate cell number of 2x10^6 NIH-3T3 cells/ml. 100 µl cells were added per 5 ml FACS tube (Falcon round-bottomed tube #352052). Cells were washed by centrifugation in 4.5 ml cold PBS at 1000 rpm for 4 minutes. Affibody molecules Z03358-NEM, Z03358-ABD-NEM, Z03358-ABD-IL2, Z02465-NEM, His_6-Z02483-NEM and negative control (GM-CSF specific Affibody molecule or *Taq*-specific Affibody molecule) were diluted in PBS + 1% FCS to 1381,6 nM and 276.3 nM (fig. 6) or 10 µg/ml (fig. 10) and 100 µl Affibody molecule sample was added per tube. After incubation for 2 hours at 4°C, cells were washed by centrifugation at 1000 rpm for 4 minutes in 4.5 ml cold PBS. Anti-Affibody antibody Ig (Birk 005) was diluted to 5 µg/ml in PBS + 1% FCS and 100 µl was added per tube. The positive control, a goat anti-PDGFR-β antibody (AF386, R&D Systems), was diluted to 5 µg/ml in PBS + 1% FCS and added to washed and unstained cells. After incubation for 1 hour at 4°C, cells were washed again by centrifugation in 4.5 ml cold PBS. Cells were then incubated with 100 µl of anti-goat-ALEXA488 (Invitrogen) diluted to 10 µg/ml in PBS + 1% FCS for 45 minutes. The samples were washed again by centrifugation in 4.5 ml cold PBS. After the last wash, 0.2 ml cold PBS was added per tube and samples were analyzed by the FACS CantoII (BD Biosciences). All results were analyzed, graphed and processed using the Microsoft Excel and GraphPad Prism[5] software.

2.5 Enzyme-linked immunosorbent assay (ELISA)

2.5.1 General ELISA protocol

A 96-well costar high binding 1/2 area plate (Corning #3690) was coated with target protein diluted in carbonate coating buffer (15 mM NA_2CO3, 35 mM $NaHCO_3$, pH 9,6) or sterile PBS (50 µl/well). After incubation overnight at 4°C, the plate was rinsed twice with tap water and blocked with PBS-C (casein, Sigma C-8654) (100 µl/well). Incubation was performed for 2 hours at RT. Affibody molecules were diluted in PBS-C and added to plate (50 µl/well) in a 2-fold serial titration. As negative control, PBS-C was used instead of Affibody molecules. After incubation for 1 hour at RT, the plate was washed

using the ELISA-washer (Skatron/Skan washer 300 (SW 300)). Goat anti-Z Ig (Adidas 001) was diluted in PBS-C (5 µg/ml) and was added to plate (50 µl/well). Also, the PDGFR-β specific positive control antibody was added to plate. The control was 2-fold serially titrated with a start concentration of 10 µg/ml. After incubation for 1 hour, the plate was washed and a rabbit-anti-goat IgG-HRP was diluted 10000x in PBS-C and added to plate (50 µl/well). After incubation for 45 minutes at RT, the plate was subsequently washed using the ELISA-washer. A TMB substrate (ImmunoPure kit, Pierce) was added to plate (50 µl/well). After incubation for 15-30 minutes at RT, the reaction was stopped with H_2SO_4 (50 µl/well). The absorbance was read using an ELISA-reader (Perkin Elmer, Victor 3).

2.5.2 ELISA variants

1. **PDGFR-β coated ELISA**

   The plates were coated with PDGFR-β at a concentration of 2 µg/ml. The Affibody molecules Z03358-NEM, Z03358-ABD-NEM, Z03358-ABD-IL2, Z02465-NEM and His_6-Z02483-NEM were added at a concentration of 34.5 nM and further diluted in a 2-fold dilution series.

2. **Specificity ELISA**

   The plates were coated with PDGFR-β, GM-CSF and VEGFR-2 all at a concentration of 2 µg/ml. The Affibody molecule Z02465-NEM was added at a concentration of 0.25 µg/ml and further diluted in a 2-fold dilution series.

3. **Effects of HSA**

   The plates were coated with 2 µg/ml PDGFR-β. The Affibody molecules Z03358-NEM, Z03358-ABD-NEM, Z03358-ABD-IL2 and Z02465-NEM were added at a concentration of 42.4 nM and further diluted in a 2-fold dilution series. All constructs, except Z03358-ABD-IL2 that already contained HSA, were tested in the absence and the presence of 5x molar excess of rHSA (Albucult, Novozymes).

All results were analyzed, graphed and processed using the Microsoft Excel and GraphPad Prism[5] software.

2.6 CTLL assay

IL-2 (human IL-2, PeproTech) was diluted in assay medium (RPMI Gibco, 10% FCS, 1% L-glutamin Lonza, 1% PEST Lonza) to 50 ng/ml. An IL-2 standard was 2-fold serially titrated in a 96-well culture plate starting at a concentration of 50 ng/ml. Four different purifications (A, B, C, D) of Affibody molecule Z03358-ABD-IL2 were diluted in assay medium to 0,2 µg/ml. 3x molar excess of rHSA was added to purification B and D and all purifications were 2-fold serially titrated in duplicates in assay medium without IL-2. As

positive controls, dilutions of IL-2 (3 ng/ml and 1,5 ng/ml) were used (100 µl/well). As negative control, assay medium was used (100 µl/well). CTLL-cells were harvested, washed and counted. Diluted in assay medium, 30000 cells/well were added to plate (100 µl/well). The plate was wrapped in plastic and incubated at 37ºC for 3 days. The reagent CCK-8 proliferation kit (Sigma) was diluted twice in assay medium and added to plate (19 µl/well). After incubation for 4 hours at 37°C, the absorbance at 450 nM was measured using an ELISA reader. All results were analyzed, graphed and processed using Microsoft Excel and GraphPad Prism[5] software.

2.7 Immunofluorescence staining of cells on glass

NIH-3T3 cells were harvested and counted. Approximately 25000 cells/well (1 drop) was added on a Multi-well slide (Histolab) using a Pasteur pipette. The slide was cultured overnight in a CO_2 incubator. The next day, culturing medium was discarded and 25µl of Z02465-Cys-NEM, His6-Z02483-NEM and negative control (*Taq*-polymerase specific Affibody molecule) diluted to a concentration of 10 µg/ml in PBS containing 1% FCS, was added per well. The slide was incubated for 3 hours at 4°C. Careful washing was done using a Pasteur pipette with cold sterile PBS. A volume of 25 µl anti-Affibody Ig (Birk 006), diluted to 5 µg/ml was added per well. As positive control, 25 µl goat α-PDGFR-β antibody diluted to10 µg/ml was added to wells with unstained cells. After incubation for one hour, slide was carefully washed in sterile PBS. A volume of 25 µl chicken α-goat IgG Alexa Fluor 488 diluted to 5 µg/ml in PBS, was added per well. Slide was incubated for 45 minutes at 4°C in darkness. The careful washing procedure with cold sterile PBS was repeated. Fixation of cells to slide was done in 3% formaldehyde (Sigma) diluted in PBS for 10 minutes at RT. Slide was washed 2x in PBS and dried. Anti-fading solution containing DAPI (Vector Laboratories) was added to dried slides. Samples were investigated using LEICA DMLA microscope equipped with a video camera.

2.8 Phosphorylation assay

An approximate cell number of 1x10^6 porcine aortic endothelial (PAE) cells, transfected with human PDGFR-β were seeded in 60-mm dishes and starved over night using F12 medium containing 1% BSA. HSA was added to His_6-Z02483-ABD-NEM at 2x molar excess. Cells were pre-incubated for 1 h at 37°C with excess at 50000x, 10000x or 2000x of Z02465-NEM, His_6-Z02483-NEM, Z02483-ABD-NEM and *Taq*-polymerase specific negative control. PDGF-BB was added to a concentration of 10 ng/ml. Two dishes with only PDGF-BB and no Affibody were used as positive controls and one dish with 150 µl sterile PBS instead of Affibody or PDGF-BB was used to see background binding. The plates were put on ice and incubated for 1 hour. After completed incubation, the medium was gently sucked away and the cells were washed twice with ice cold PBS.
Cells were detached by adding 2 ml of PBS per dish and using a cell scraper to loosen the cells from plastic. The solution was then transferred to 10 ml falcon tubes.

To obtain a dry pellet, the tubes were centrifuged for 3 minutes at 1000 rpm at 4°C. The supernatant was gently removed using a Pasteur pipette. The cell pellets were lysed in 100 µl lysing buffer (20 mM EDTA, 137 mM NaCl, 20 mM Tris (pH 8.0), 1% NP-40, 10% glycerol) with the addition of Complete EDTA-free (Roche) plus PhosStop phospatase inhibitor (Roche). The pellet was dissolved carefully and transferred to eppendorf tubes. The samples were incubated in cold room for 30 minutes and then centrifuged for 15 minutes at 13000 rpm. The supernatant was transferred to clean tubes and stored at -80°C until use.

DuoSet® IC was used in order to measure PDGFR-β in cell-lysates. The capture antibody was diluted to a concentration of 6 µg/ml in PBS and a 96-well microplate was coated with 50 µl per well. The plate was wrapped in plastic and left overnight at RT. The plate was washed twice with water. A volume of 100 µl block buffer (1% BSA, 0.05% NaN_3 in PBS, pH 7.2-7.4) was added to plate. After blocking at RT for 1.5 hours, the plate was washed using ELISA washer. Samples were added at a volume of 50 µl. The plate was wrapped in plastic and incubated for 2 hours at RT. After wash, the detection antibody was diluted to 500 ng/ml in IC Diluent#14 (20 mM Tris, 137 mM NaCl, 0.5% Tween 20, 0.1% BSA, pH 7.2-7.4) and added at a volume of 50 µl/well. The plate was wrapped in plastic and incubated for 2 hours at RT. The plate was again washed and Streptavidin-HRP was diluted in IC Diluent#14 1100 times and added at 50 µl/well. After incubation for 40 minutes in RT the washing step was repeated. Substrate solution was added at 50 µl/well and the plate was incubated at 20 minutes in RT. Stop solution was added at 50 µl to each well. The absorbance was read using an ELISA-reader (Perkin Elmer, Victor 3) set to 450 nm. All results were analyzed, graphed and processed using Microsoft Excel.

The phosphorylation assay was done in collaboration with Eliane Cortez at the department of medical biochemistry and biophysics (MBB) at KI and the ELISA was done in collaboration with Ingmarie Höidén-Guthenberg at Affibody AB.

3. Results

Prior to this degree project, the Affibody molecule Z02465 specific for PDGFR-β was selected from an Affibody library based on the original scaffold. The scaffold has since then been altered with the aim to improve thermal stability and to get a more hydrophilic surface. The binding sequence of Z02465 was grafted into the new scaffold, thereby creating the PDGFR-β specific Affibody molecule Z03358. The Z03358 was fused with ABD and IL-2 to allow for targeted anti-cancer therapy. Unexpectedly, the grafting of the binding sequence to the new scaffold affected the binding ability of Z03358. Therefore, increased attention was directed at two PDGFR-β specific Affibody molecules based on the original scaffold: the PDGFR-β specific Affibody molecule Z02465 and the PDGFR-β specific Affibody molecule Z02483, a variant of Z02465. The characterisation of Affibody molecule fusion Z03358-ABD-IL2 is presented first, followed by a characterisation and a comparison of Affibody molecules Z02465 and Z02483.

3.1 Z03358-ABD-IL2

The aim of this part of the project was to investigate if all three constituents of Z03358-ABD-IL2 were functional after fusion. First of all, control Affibody molecules Z03358-cys and Z03358-ABD-cys were NEM-conjugated. The controls were included in experiments in order to see how each part of the construct functioned individually. Also, Z02465 and Z02483 were included in experiments in order to compare binding ability of the new (Z03358) and the old scaffold (Z02465). The ability of Z03358-ABD-IL2 to bind PDGFR-β and PDGFR-β expressing cells, was analyzed using ELISA and flow cytometry. The effect of human serum albumin (HSA) was investigated, because of the albumin-binding domain (ABD) that was included in the construct to increase the half-life of the compound. At last, by performing two cell assays using an IL-2 dependent cytotoxic T-cell line, it was investigated whether IL-2 was biologically active in Z03358-ABD-IL2.

3.2 NEM-conjugation of two Affibody molecules used as controls

Two control Affibody molecules were included in all flow cytometry and ELISA experiments performed: Z03358-NEM and Z03358-ABD-NEM. Since both control Affibody molecules were cys-tagged at the beginning (Z03358-cys and Z03358-ABD-cys), they had to be N-ethyl maleimide (NEM)-conjugated before usage. The NEM-conjugation was done in order to prevent free cysteines of Affibody molecules from binding to each other, thus affecting the results. The NEM-conjugated proteins were analyzed on a SDS-PAGE gel in order to confirm expected mass spectrometry results of both constructs. In fig. 3, a SDS-PAGE of Z03358-ABD-NEM is shown in its non-reduced, reduced and NEM-conjugated form (fig. 3: b, d, f). The results show that both Z03358-ABD-cys and Z03358-cys (data not shown) were successfully NEM-conjugated.

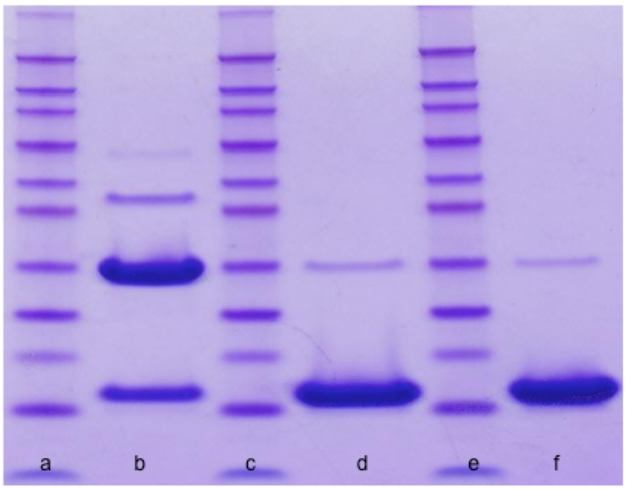

Figure 3. SDS-PAGE of NEM conjugated Z03358-ABD-cys. The samples were as follows: a) ladder b) unreduced Z03358-ABD-cys c) ladder d) reduced Z03358-ABD-cys e) ladder f) NEM-conjugated Z03358-ABD-cys. The result in f shows a successfully NEM-conjugated Affibody molecule with only a small amount of unreduced Z03358-ABD-cys.

3.3 Characterisation of Z03358-ABD-IL2 by ELISA

An ELISA was performed in order to study the ability of Z03358-ABD-IL2 to bind to PDGFR-β. The experiment was performed twice in order to confirm the results. Fig. 4 shows the difference in target binding ability between tested constructs. EC_{50}-values in table 1 based on curve-fits shown in fig. 5 showed that Z03358 bound to PDGFR-β, but with lower affinity than Z02465-NEM and His_6-Z02483-NEM. Among Z03358 constructs, Z03358-ABD-NEM showed a slightly better affinity to PDGFR-β than Z03358-NEM. Z03358-ABD-IL2 was ranked lowest in both experiments. The anti-PDGFR-β antibody gave a strong signal. Ranking within each experiment was the same.

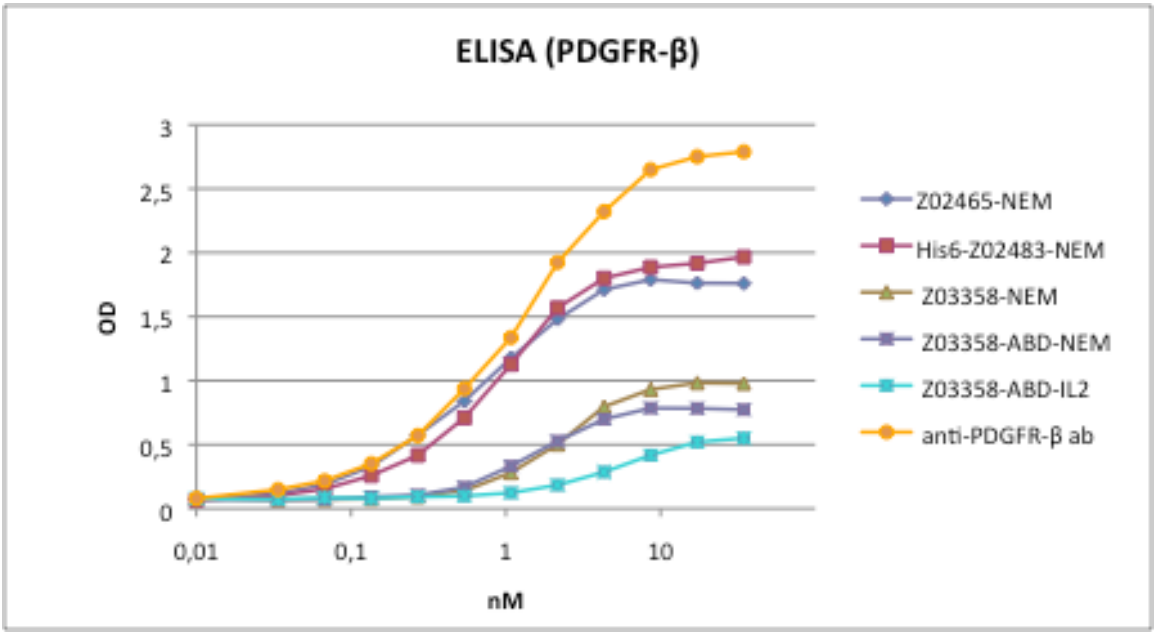

Figure 4. ELISA analysis of the PDGFR-β specific Affibody molecules to PDGFR-β. The analysis shows differences in target binding ability. The anti-PDGFR-β antibody was used as positive control. Affibody molecule Z02465-NEM and His_6-Z02483-NEM showed a strong ability to bind PDGFR-β. Affibody molecule Z03358-ABD-IL2 and positive controls Z03358-NEM and Z03358-ABD-NEM showed a less strong binding ability. The graph shows optical density versus concentration (nM). The Z03358-constructs were 2-fold serially titrated.

Table 1. EC_{50}-values in nM calculated from curve-fits in fig.5 by GraphPad Prism[5] software. The table shows a comparison in binding ability to PDGFR-β with the best binding ability ranked as number one.

	EC_{50} (nM)
1. Z02465-NEM	0,6
2. His_6-Z02483-NEM	0,9
3. Z03358-ABD-NEM	1,5
4. Z03358-NEM	2,2
5. Z03358-ABD-IL2	3,7

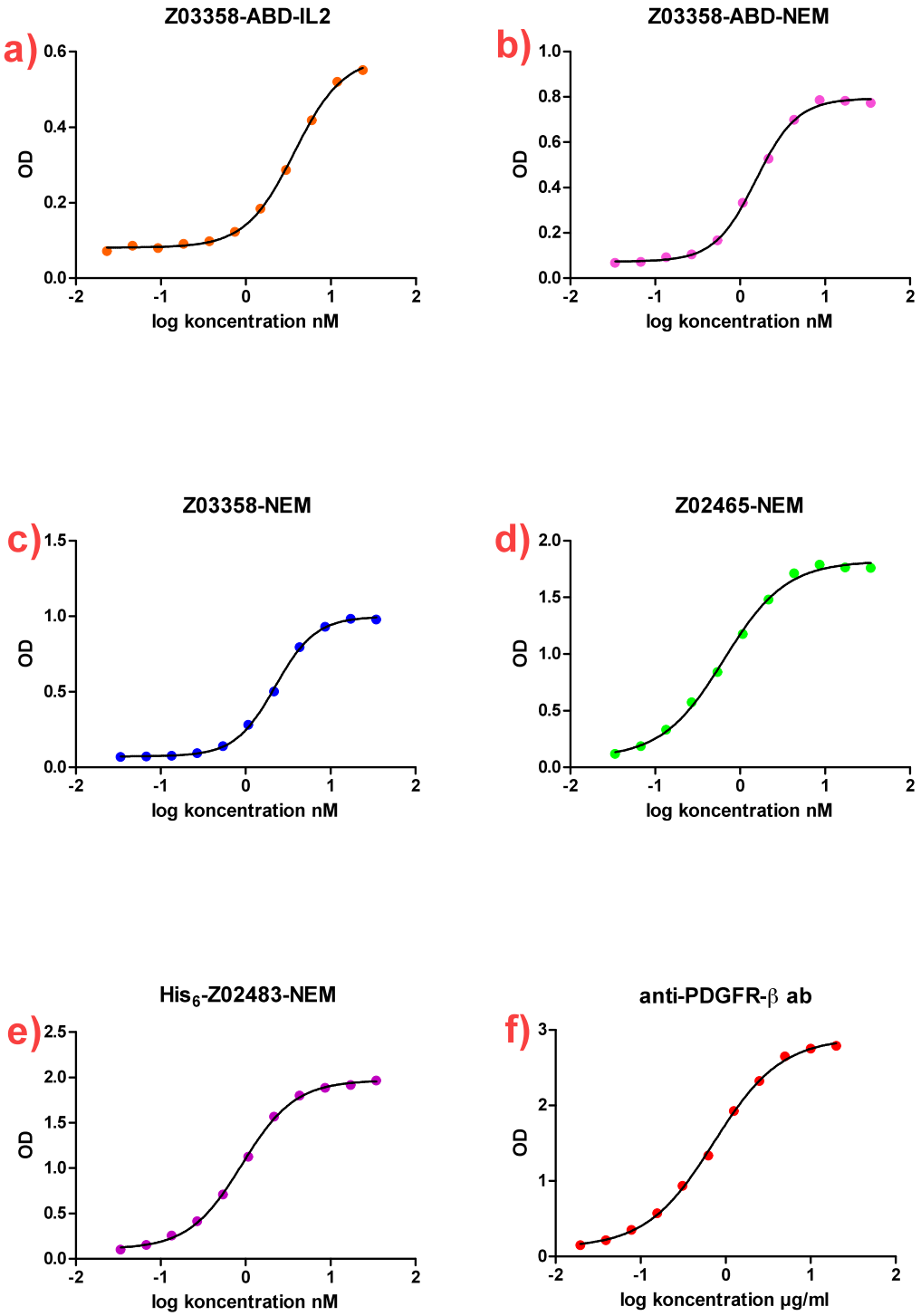

Figure 5. Curve-fits calculated by GraphPad Prism[5] software based on ELISA analysis of the PDGFR-β specific Affibody molecules: a) Z03358-ABD-IL2 b) Z03358-ABD-NEM c) Z03358-NEM d) Z02465-NEM e) His_6-Z02483-NEM f) anti-PDGFR-β antibody. The graphs show optical density versus log concentration (nM). The Z03358-constructs were 2-fold serially titrated. EC_{50}-values are shown in table 1.

3.4 Effects of HSA on the binding capacity of Z03358-ABD-IL2

To investigate if human serum albumin (HSA) affected the affinity of Z03358 constructs to PDGFR-β, an ELISA was performed in the presence of 5x molar excess of HSA. Results presented in table 2, showed that the addition of HSA did not affect binding of Z03358-ABD-NEM to PDGFR- β. Z03358-ABD-IL2 already contained HSA and was only included in the experiment in order to see how it was ranked in comparison to positive control Z02465 and Z03358-controls.

Table 2. EC_{50}-values in nM based on an ELISA analysis. Results were calculated by GraphPad Prism[5] software. The table shows a comparison in binding ability to PDGFR-β between Z03358-ABD-NEM in the presence or absence of HSA. Affibody molecule Z02465-NEM and Z03358-NEM is used as controls. Construct with best binding ability to PDGFR-β is ranked as number one.

	EC_{50} (nM) -HSA	EC_{50} (nM) +HSA
Z02465-NEM	0,1	0,2
Z03358-ABD-NEM	0,6	0,6
Z03358-NEM	0,8	1,2
Z03358-ABD-IL2 purification A		1,4

3.5 Characterisation of Z03358-ABD-IL2 by flow cytometry

In order to give answer to the question whether switch from old scaffold (Z02465) to new scaffold (Z03358) affected binding ability to PDGFR-β expressing cells, flow cytometry analyses were performed. The results in fig. 6 were confirmed in several experiments and showed that Z03358 constructs gave a much lower signal than positive controls Z02465-NEM, His_6-Z02483-NEM and the PDGFR-β specific antibody. Compared to Z03358-NEM, Z03358-ABD-NEM showed a slightly better binding ability to NIH-3T3 cells. This indicated that fusion with ABD did not affect binding properties of the molecule. The results were comparable to ELISA-results, thus suggesting that the low binding ability of Z03358 constructs to PDGFR-β expressing cells was because of the new scaffold. Taken together, the experiment showed that Z03358-ABD-IL2 did not have sufficient affinity to NIH-3T3 cells in order to function as a therapeutic agent. The results showed a concentration-dependent binding.

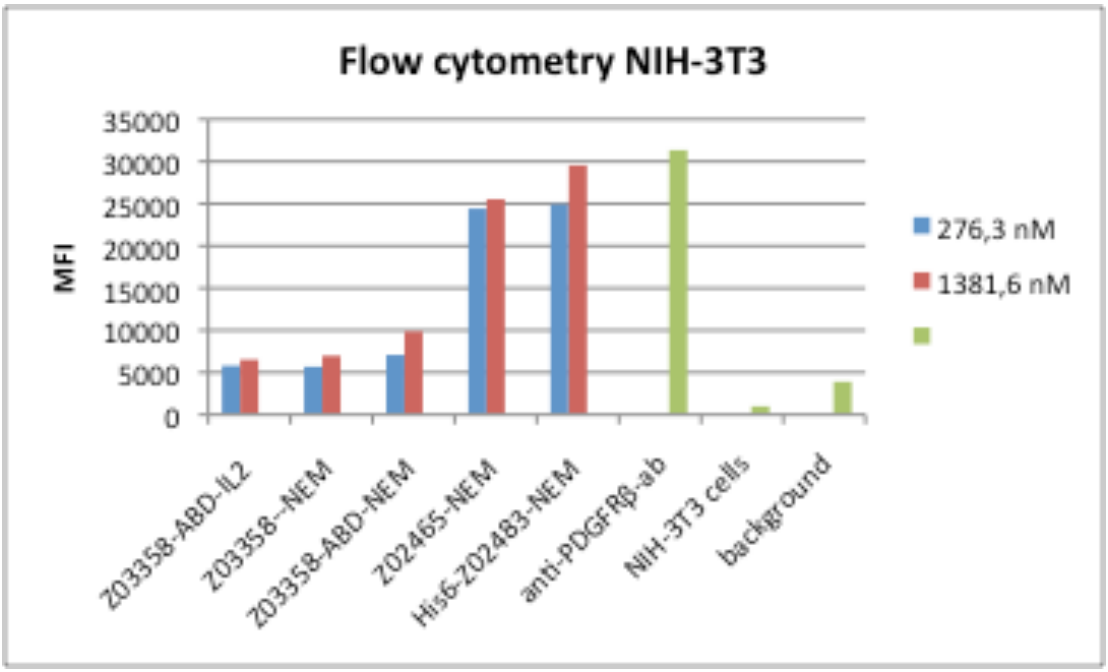

Figure 6. Flow cytometry analysis of PDGFR-β specific Affibody molecules to PDGFR-β expressing NIH-3T3 cells. Affibody molecules His_6-Z02483-NEM, Z02465-NEM and the anti-PDGFR-β antibody used as positive control showed a strong binding ability to NIH-3T3 cells. Affibody molecule Z03358-ABD-IL2 and positive controls Z03358-NEM and Z03358-ABD-NEM showed a less strong binding ability. Cells were stained with respective Affibody molecule as described in materials and methods. The results are shown as mean fluorescence intensity (MFI).

3.6 Functionality of IL-2 in the Z03358-ABD-IL2 fusion protein

To see if the IL-2 protein in Z03358-ABD-IL2 was bioactive in four different purifications of Z03358-ABD-IL2, CTLL-assays were performed (fig. 7). Experiments done showed that purification A-C contained active IL-2, while purification D had a very low activity (table 3). The conclusion was that purification D gave the least active IL-2 whereas more experiments would be needed to verify the difference between purifications A-C.

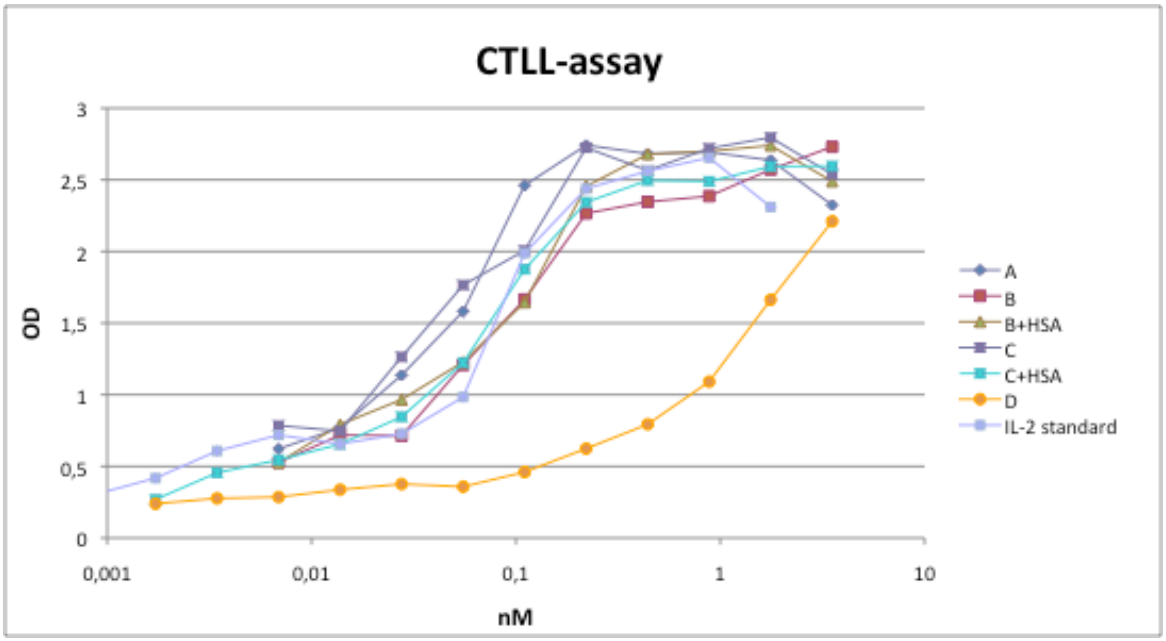

Figure 7. The IL-2 activity of Z03358-ABD-IL-2 was tested using the IL-2 dependent cytotoxic T-cell line (CTLL) in a CTLL-assay. The experiment showed that Z03358-ABD-IL2 purifications A-C contained fully bioactive IL-2 compared to the IL-2 standard. Purification D showed a low activity. The experiment was repeated with approximately the same results.

Table 3. Activity in % of Z03358-ABD-IL2 purification A-D. The result showed that construct A-C contained active IL-2. Purification D showed a low activity. No conclusions could be drawn regarding best activity between purification A-C or between purifications in the presence of absence of HSA.

	Exp1 activity (%)	Exp2 activity (%)
A	151,3	134,5
B	-	66,6
B+HSA	-	69,4
C	91,4	-
C+HSA	-	71,2
D	5,7	4

3.7 SDS-PAGE analysis of PDGFR-β binders

To exclude presence of degradation products affecting the results, a SDS-PAGE gel was done with all constructs used (fig. 8). The conclusion drawn from coomassie stained SDS-PAGE gel results is that all Affibody molecules used during the project were in proper condition and were not affected by storage. Also, all Affibody molecules used as negative controls were in proper condition (data not shown).

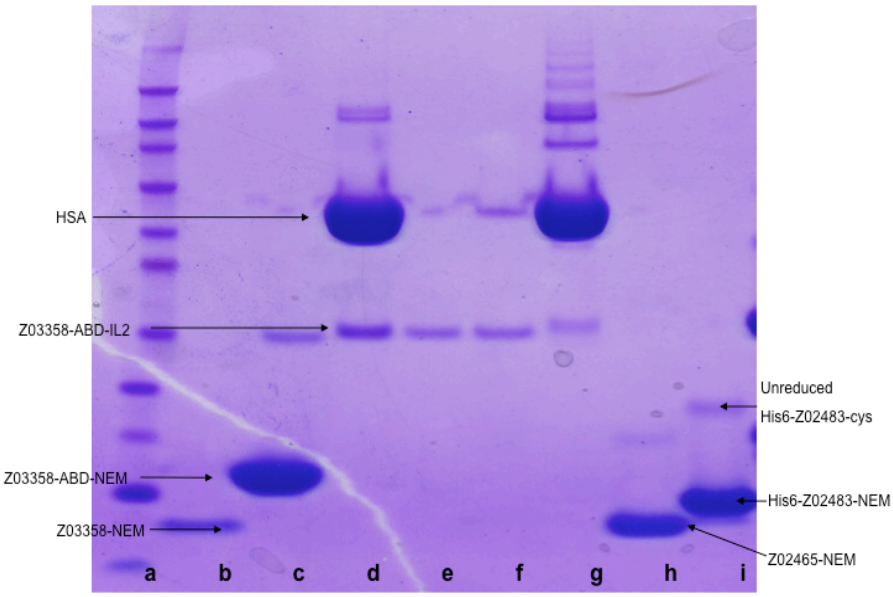

Figure 8. SDS-PAGE of Affibody molecules used in the thesis. The samples were as follows: a) ladder b) Z03358-NEM c) Z03358-ABD-NEM d) Z03358-ABD-IL2 purification A + HSA e) Z03358-ABD-IL2 purification B f) Z03358-ABD-IL2 purification C g) Z03358-ABD-IL2 purification D + HSA h) Z02465-NEM i) His_6-Z02483-NEM. The SDS-PAGE showed that all Affibody molecules were in proper condition.

3.8 Conclusions Z03358-ABD-IL2

Taken together the results showed that Z03358-ABD-IL2 had a great potential by its ability of activating cytotoxic T-cells, but the grafting of the binding epitope in Z02465-NEM from the old to the new scaffold caused a decreased PDGFR-β binding capacity. Importantly, it was also shown that human serum albumin did not affect the binding ability of the construct.

3.9 Z02465 and Z02483

Since the target binding criteria of Z0335-ABD-IL2 was not fulfilled, attention was put on the two control Affibody molecules consisting of the original scaffold: Z02465-NEM and His_6-Z02483-NEM. The aim was to compare and characterise the two candidates in order to choose the best one to go further with in case of a scaffold change of the IL-2 fusion protein. ELISA and flow cytometry was performed in order to find out which molecule had best binding ability to PDGFR-β and to PDGFR-β expressing cells. Also, because of the potential use of the molecules as antagonists in humans it was interesting to investigate whether both constructs could block PDGF-BB from binding to PDGFR-β. This was done in a phosphorylation assay.

3.10 Comparison of Z02465 and Z02483 by ELISA

ELISA was used in order to compare affinity of Z02465-NEM and His_6-Z02483-NEM to PDGFR-β. Two experiments were done in the same way. EC_{50}-values in table 4 based on curve-fits shown in fig. 9 (for experiment one) showed that Z02465-NEM had a slightly better affinity to PDGFR-β than His_6-Z02483-NEM. Z03358-NEM was included in the experiment in order to confirm previous results.

Table 4. EC_{50}-values in nM. Results in column 1 are calculated from curve-fits in fig.9 by GraphPad Prism[5] software. The table shows a comparison in binding-ability to PDGFR-β. Affibody molecule with the best binding ability is ranked as number one.

	1: EC_{50} (nM)	2. EC_{50} (nM)
Z02465-NEM	0,9	0,6
His_6-Z02483-NEM	1,0	0,9
Z03358-NEM	4,4	2,2

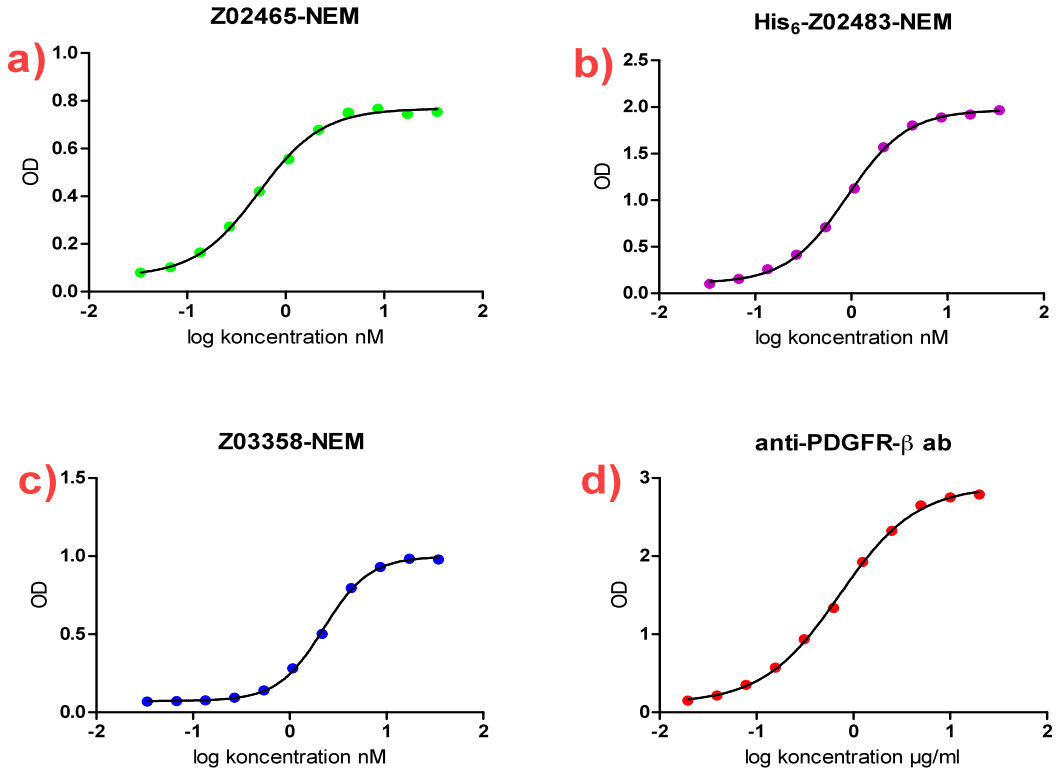

Figure 9. Curve-fits calculated by GraphPad Prism[5] software based on ELISA analysis of the PDGFR-β specific Affibody molecules a) Z02465-NEM b) His_6-Z02483-NEM c) Z03358-NEM d) anti-PDGFR-β antibody. The graphs show optical density versus log concentration (nM). Constructs were 2-fold serially titrated. EC_{50}-values are shown in table 4, column one.

3.11 Comparison of Z02465 and Z02483 by flow cytometry

The binding capacity of Z02465-NEM and His_6-Z02483-NEM to NIH-3T3 cells was compared using flow cytometry. The results shown in fig. 10 were confirmed in several experiments. Z03358-NEM was only included in the experiment in order to confirm previous results. As shown in fig. 10 His_6-Z02483-NEM had a better affinity to NIH-3T3 cells than Z02465-NEM. Z03358-NEM showed a small signal. The anti-PDGFR-β antibody gave a strong signal, although not as strong as His_6-Z02483-NEM in this experiment. Signal from all constructs was concentration dependent. Results were opposite from ELISA-results where His_6-Z02483-NEM was the best binder (fig. 9).

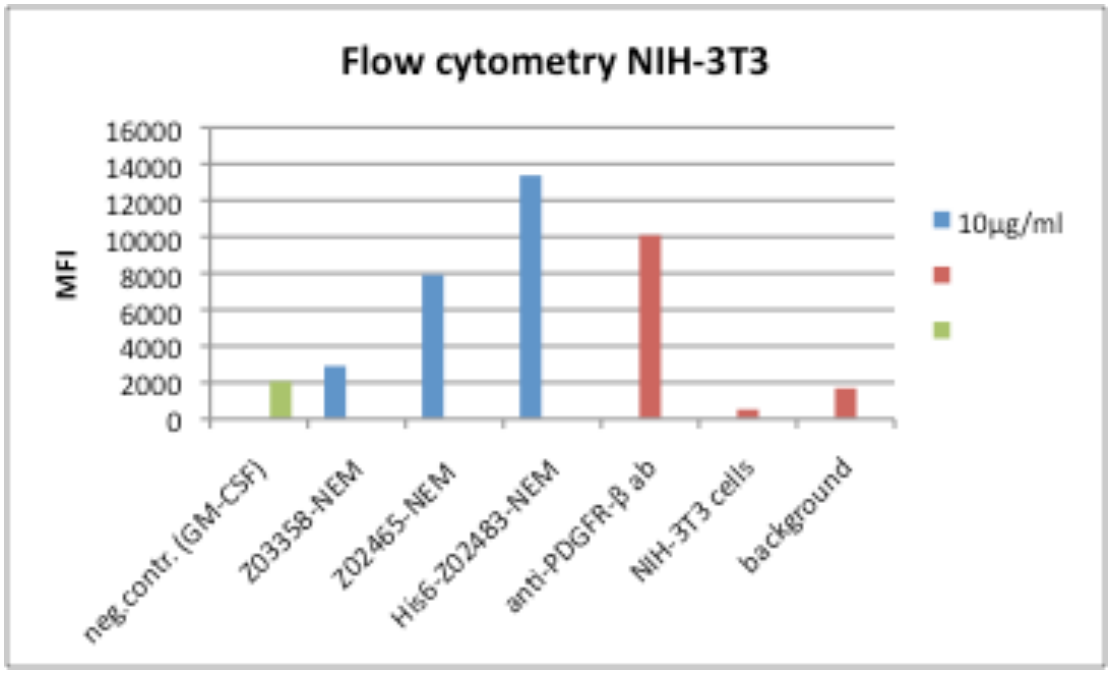

Figure 10. Flow cytometry analysis of the PDGFR-β specific Affibody molecules to PDGFR-β expressing NIH-3T3 cells. Affibody molecules His_6-Z02483-NEM and Z02465-NEM and the anti-PDGFR-β antibody used as positive control showed a strong binding ability to NIH-3T3. Affibody molecule Z03358-ABD-IL2 and positive controls Z03358-NEM and Z03358-ABD-NEM showed a less strong binding ability to NIH-3T3 cells. Cells were stained with respective Affibody molecule as described in materials and methods. The results are shown as mean fluorescence intensity (MFI).

3.12 Immunofluorescence staining of Z02465 and Z02483

Immunofluorescence staining with Z02465-NEM and His_6-Z02483-NEM was made in order to verify membrane binding to NIH-3T3 cells. The results in fig. 11 showed slightly more intense staining of His_6-Z02483-NEM compared to Z02465-NEM. The Affibody molecule Z03358 did not stain NIH-3T3 cells much and was comparable to negative control (data not shown). Results supported previous FACS results, ranking His_6-Z02483-NEM as the best binder to NIH-3T3 cells. The positive control, anti-PDGFR-β antibody, showed a strong staining of cells.

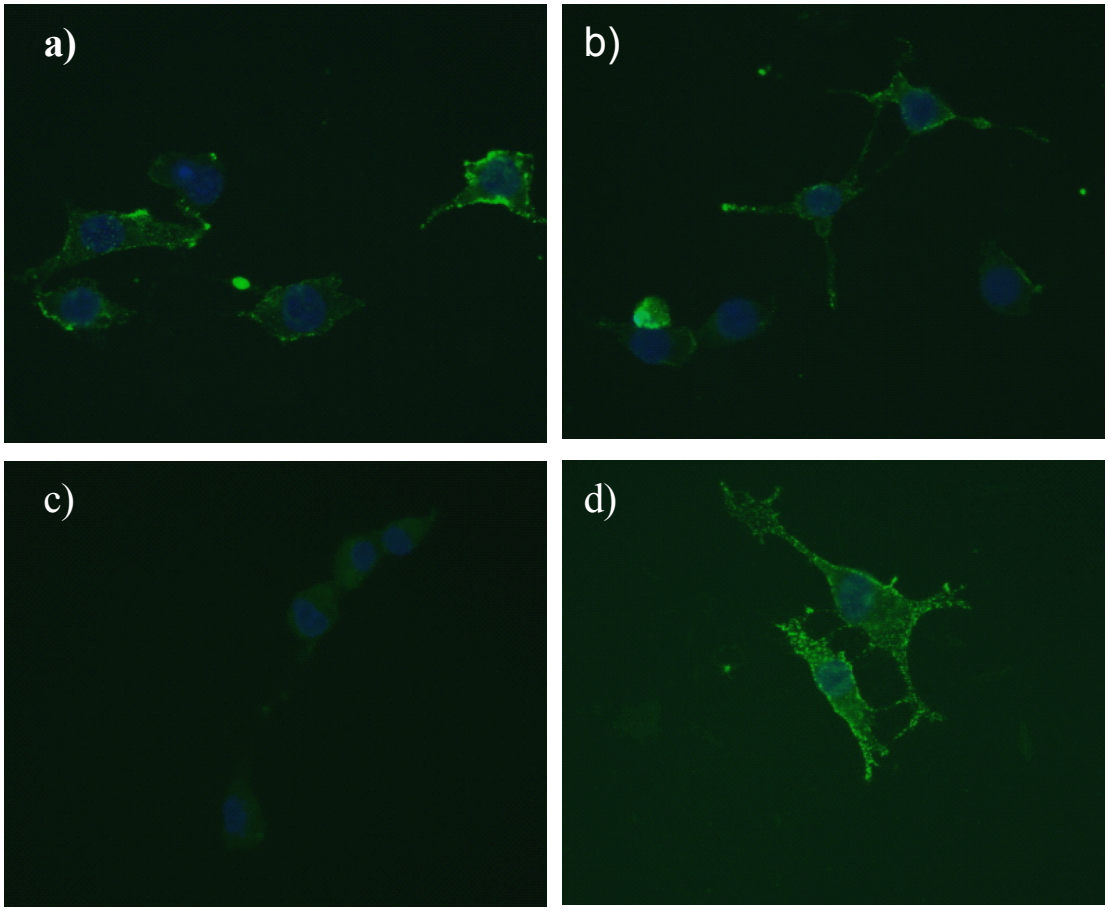

Figure 11. Immunofluorescence staining of PDGFR-β expressing NIH-3T3 cells with a) His_6-Z02483-NEM b) Z02465-NEM c) background d) anti-PDGFR-β antibody. The cells were stained as described in materials and methods.

3.13 Phosphorylation assay

The constructs were tested for blocking ability of PDGF-BB induced phosphorylation of porcine aortic endothelial (PAE)-cells. Results in fig. 12 showed that both Z02465-NEM and His_6-Z02483-NEM blocked phosphorylation in a concentration dependent manner. The ABD fused Z02483 showed a somewhat weaker ability to block phosphorylation. No blocking was seen, neither in the two positive controls including only PDGF-BB nor the irrelevant *Taq*-specific Affibody molecule Z04726 used as negative control. The PBS-control without PDGF-BB or Affibody showed the background signaling.

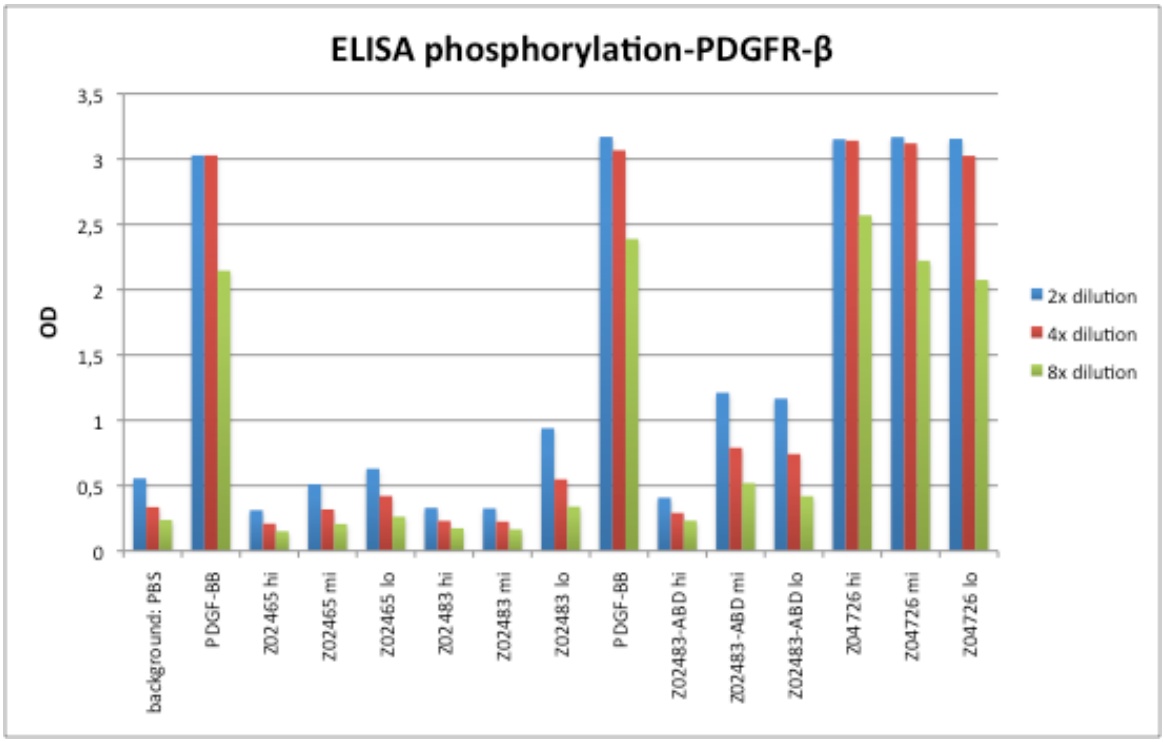

Figure 12. Phosphorylation assay with Affibody molecules Z02465-NEM, His_6-Z02483-NEM and Z02483-ABD-NEM using PDGFR-β expressing PAE-cells. Diagram shows optical density of the samples. Various Affibody molecule excess is marked with hi, mi or lo. Negative control with PBS shows background binding. Positive controls show PDGF-BB induced phosphorylation. See material and methods.

3.14 Specificity testing of Z02465 by ELISA

Even though Z02483 did show slightly better binding ability to PDGFR-β expressing cells, Z02465 was a safer choice to continue with since it already had been tested in animals with good targeting results. Therefore, an extra specificity test was done with this candidate. The Affibody molecule was tested against three different targets: GM-CSF, VEGFR-2 and PDGFR-β. The Affibody molecule Z02465-NEM was titrated in a two-fold serial dilution and was analyzed against the three targets. As shown in fig. 13, no unspecific binding could be seen of Z02465-NEM.

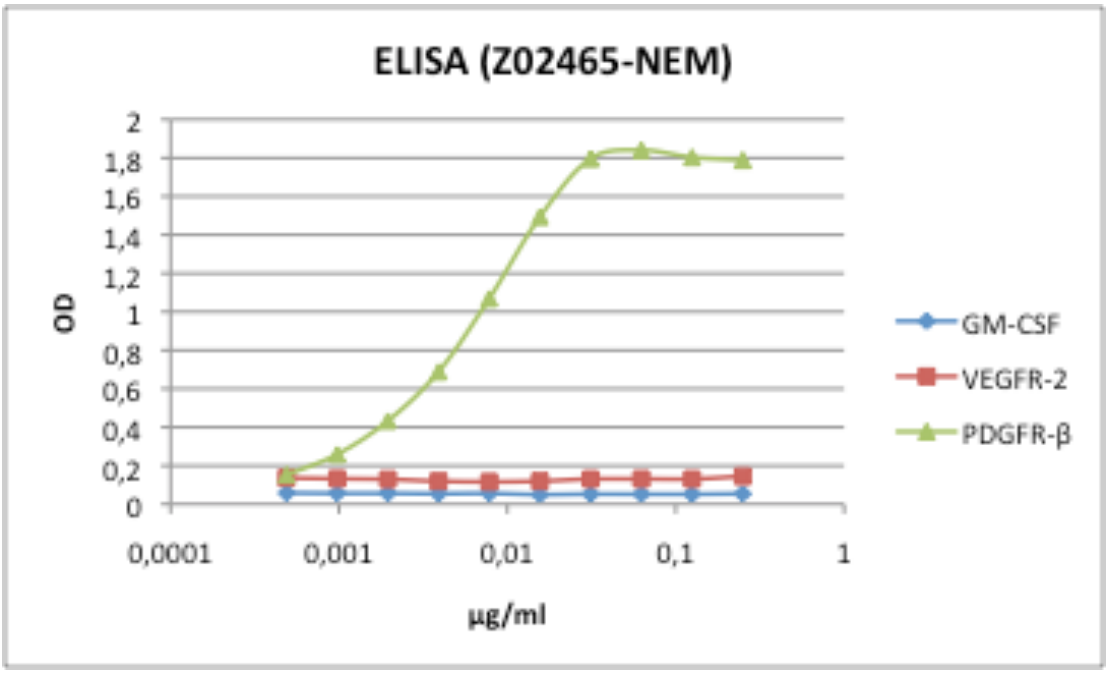

Figure 13. ELISA analysis of the PDGFR-β specific Affibody molecule Z02465-NEM to target GM-CSF, VEGFR-2 and PDGFR-β. The results confirm that Z02465-NEM is specific to PDGFR-β. The graphs show optical density versus log concentration (µg/ml). Constructs were 2-fold serially titrated.

3.15 Conclusions of Z02465 versus Z02483

Taken together, the results showed that His_6-Z02483-NEM had a better binding ability to PDGFR-β expressing NIH-3T3 cells compared to Z02465-NEM. These results were opposite to results in ELISA where Z02465-NEM showed a slightly better affinity to PDGFR-β compared to His_6-Z02483-NEM. It has also been shown that both constructs block phosphorylation of PAE-cells.

4. Discussion

This work was part of a research program with the overall aim of concentrating immuno-stimulatory molecules in tumour lesions in order to elicit therapeutic antitumour responses. The specific aim of the degree project involved *in vitro* characterisation of target binding and IL-2 activity of a PDGFR-β specific fusion protein aimed for vascular targeted tumour therapy. The fusion protein (based on the new Affibody scaffold Z03358) was compared to the corresponding binder in the old scaffold (Z02465) and tested for target binding using flow cytometry and ELISA analysis. The results showed that grafting the epitope from old to new scaffold, in some way had affected cell binding properties negatively of the Affibody molecule. This was surprising and unexpected since the Affibody molecule Z03358 and Z02465 displayed similar affinity to PDGFR-β in previous Biacore analyses. Also, extensive analysis in order to identify amino acids that might be replaced without disturbing the overall structure had been done, but clearly, the old scaffold variant Z02465 was superior in terms of cellular binding. However, it was shown in ELISA and flow cytometry that Z03358 constructs did show some binding-ability to PDGFR-β, although with much lower signals than Z02465 or the PDGFR-β specific antibody. In flow cytometry analysis, it was challenging to establish a stable negative control. Initially the *Taq*-specific Affibody molecule was used but since it fluctuated unacceptably between the experiments (probably due to ”stickiness”) we tested a GM-CSF specific Affibody molecule, which showed less unspecific binding (fig. 9). Thus, because of the unacceptably high and unstable background binding, the negative control is not shown in fig. 10. The reason for not repeating the experiment was because of the limited amount of some of the samples and since it was obvious, based on previous results, that the only inadequate result was the signal of the negative control. However, Z02465 and Z02483 had much higher signal than background and it was clear that Z03358 did not have the same binding ability compared to the old scaffold.

In order to analyze for IL-2 activity of the construct, a CTLL-assay was established. Four different purifications (A, B, C, D) of Z03358-ABD-IL2 (purified by Per Jonasson at Affibody AB) were tested twice in two identical assays in order to confirm IL-2 induced proliferation. The reason for purifying the construct in four different ways was because of the difficulties regarding retained IL-2 activity during the purification process. Therefore, it was important to determine which purification protocols that yielded fully active IL-2. The results showed that the IL-2 part of the construct had full activity, but since the PDGFR-β binding part of Z03358-ABD-IL2 was too low, a new clone of the fusion protein based on the best PDGFR-β binder in the old scaffold would be needed in order to succeed.

For this reason, we further analyzed functions of Z02465 and Z02483. Interestingly, the ability to bind differed somewhat when using various methods. In ELISA, Z02465 showed a slightly better binding ability to PDGFR-β than Z02483, whereas in flow cytometry Z02483 showed a better binding ability than Z02465. The most likely reason

for this may be due to the dissimilar conditions of the two methods and this also points at the importance of testing Affibody molecules with different methods in order to come to accurate conclusions.

Attempts were also made to develop a PDGF-BB blocking assay in ELISA, but binding between PDGFR-β and PDGF-BB could not be established and the right conditions for making the blocking experiment to work were not found. However, since a cell based assay would be biologically more relevant, cell-signaling studies were prioritized.

Even though receptor blocking capability is likely not a major factor influencing the therapeutic success of the fusion protein, as the main task is to concentrate IL-2 to the tumour vasculature, it was interesting (also in case of additional therapeutic areas) to see whether Z02465 and Z02483 could function as antagonists and block PDGF-BB from binding PDGFR-β and block ligand induced phosphorylation in cells. Therefore a phosphorylation assay was done. The phosphorylation assay was performed at KI, where PDGFR-β transfected PAE cells were used. The use of this cell-line instead of NIH-3T3 cells was preferable since NIH-3T3 cells express both PDGFR-β and PDGFR-α and the PDGFR-α is also binding PDGF-BB. PDGF-BB binding of PDGFR-α would probably affect the results of the experiment. After immunoprecipitation of the receptor, the phosphorylation status was analyzed by ELISA. To be sure to succeed with the blocking and based on results from a previous study of Z02465[7] where phosphorylation status was analyzed by Western blot, a large excess of Affibody molecules were used when staining the cells. The large excess of Affibody molecules was not directly correlating with the low KD-value of the Affibody molecule in Biacore. The Affibody molecule excess required in order to block PDGF-BB from binding PDGFR-β in a cell-assay, includes many possible explanations. One likely reason could be that PDGF-BB is a dimer, thus having two binding sites to the dimeric receptor, in contrast to the Affibody molecule, which only has one binding site. Therefore it seems reasonable that PDGF-BB, in some way, is superior the Affibody molecule to bind PDGFR-β. The results of the experiment showed convincingly that both Z02465 and Z02483 could block PDGF-BB induced phosphorylation at 2000x molar Affibody molecule excess (the lowest dose tested). When looking at the results of the ABD fused variant of Z02483, the construct blocked phosphorylation, but maybe not as well as Z02465 and Z02483. The reason for this could be because of steric hindrance or that the added human serum albumin affected the Affibody molecule binding negatively, but the experiment is only done once and it has to be repeated in order to draw any conclusions. However, the phosphorylation assay shows promising results that further support the use of Z02465 or Z02483 in future therapy experiments.

5. Overall conclusion and future perspectives

To summarize, the results showed that Z03358-ABD-IL2 had activity in all parts, but that the grafting of the binding epitope in Z02465 from the old to the new scaffold caused a decreased PDGFR-β binding capacity. Importantly, it was also shown in ELISA that the binding of ABD fused construct was not affected by the addition of human serum albumin, thus suggesting that the format of the molecular construct is functional, but that the PDGFR-β targeting moiety Z03358 was suboptimal. It has also been shown that Affibody molecules Z02465-NEM and His_6-Z02483-NEM do have a good target binding function both in flow cytometry and ELISA and can function as antagonists and block PDGF-BB induced phosphorylation. Taken together, the findings suggests that a change of scaffold Z03358 should be considered, since the immunocytokine was active and the only criteria not fulfilled was the target binding function which, convincingly shown, was due to the scaffold. Previous studies have also shown that Z02465-NEM does function in vivo, and it can be concluded that Z02465-NEM should be a good candidate for targeting IL-2 to tumour vasculature.

However, if scaffold change was to be done, there are some more issues to investigate in order to perform a full pre-study characterisation. The experiments listed below are important investigations to do and were left out from this project work because of the unfilled target binding criteria of Z03358-ABD-IL2.

- NK-cell cytotoxicity assay
- Biodistribution analysis
- Animal therapy

6. Acknowledgements

First of all, I would like to give a special and warm thank you to my engaged and supportive supervisors Ingmarie Höidén-Guthenberg and Fredrik Frejd at Affibody AB for being the best supervisors one can ask for. I also want to thank my opponent Thomas Läräng and my scientific reviewer Angelica Loskog at the department of Immunology, Genetics and Pathology (IGP) at Rudbeck laboratory, for doing a great job with the last finish. I want to thank Eliane Cortez and Kristian Pietras at the department of medical biochemistry and biophysics (MBB) at KI for a good collaboration doing the phosphorylation-assay. I want to thank Olof Widmark at Affibody for showing me how to NEM conjugate and to do the SDS-PAGE. Thanks also to Gunilla Fant and Malin Lindborg; it has been a pleasure having you as roommates. At last, thank you to all nice people working at Affibody AB, I have really appreciated the positive and nice atmosphere while being here.

8. References

1. Campbell, Neil A & Reece, Jane B: The molecular biology of cancer: Campbell, Neil A & Reece, Jane B. (red), *Biology* 2002, 368-372. United states of America: Pearson Education.

2. Joyce JA: Therapeutic targeting of the tumour microenvironment. Cancer cell 2005, 7:513-520

3. Carmeliet P: Angiogenesis in health and disease. Nat Med 2003, 9:653–660

4. Bergers G, Song S: The role of pericytes in blood-vessel formation and maintenance. Neuro-oncology 2005, 7:452-464

5. Morikawa S, Baluk P, Kaidoh T, Haskell A, Jain RK, McDonald DM: Abnormalities in pericytes on blood vessels and endothelial sprouts in tumours. Am J Pathol 2002, 160(3); 985-1000

6. Östman A, Heldin C-H: PDGF receptors as targets in tumor treatment. Adv Cancer Res 2007, 97: 247–274.

7. Lindborg M, Cortez E, Höiden-Guthenberg I, Gunneriusson E, von Hage E, Syud F, Morrison M, Abrahmnsén L, Herne N, Pietras K, Frejd FY: Engineered high-affinity Affibody molecules targeting platetelet-derived growth factor receptor β *in vivo*. J Mol Biol 2011, doi: 10.1016/j.jmb.2011.01.033

8. Pietras K, Östman A: Hallmarks of cancer: Interactions with the tumor stroma. Exp Cell Res 2010, doi: 10.1016/j.yexcr.2010.02.045

9. Shen J, Vil MD, Prewett M, Damoci C, Zhang H, Huiling L, Jimenez X, Deevi D S, Lacolina M, Kayas A, Bassi R, Persaud K, Rohoza-Asandi A, Balderes P, Loizos N, Ludwig DL, Tonra J, Witte L, Zhu Z: Development of a fully human anti-PDGFRβ antibody that suppresses growth and enhances antitumor activity of an anti-VEGFR2 antibody. Neoplasia 2009, 11: 594-604

10. Shen J, Vil MD, Zhang H, Tonra JR, Rong L, Democi C, Prewett M, Deevi DS, Kearney J, Surguladze D, Jimenez X, Lacolina M, Bassi R, Zhou K, Balderes P, Mangalampalli VR, Loizos N, Ludwig DL, Zhu Z: An antibody directed against PDGF receptor beta enhances the antitumor and the anti-angiogenic activities of an anti-VEGF receptor 2 antibody. Biochem Biophys Res Commun 2007, 357:1142.1147

11. Timke C, Zieher H, Roth A, Hauser K, Lipson KE, Weber KJ, Debus J, Abdollahi A, Huber PE: Combination of vascular endothelial growth factor receptor/platelet-

derived growth factor receptor inhibition markedly improves radiation tumor therapy. Clin Cancer Res 2008, 14(7)

12. Pietras K, Rubin K, Sjöblom T, Buchdunger E, Sjöquist M, Heldin CH, Östman A: Inhibition of PDGF receptor signaling in tumour stroma enhances antitumor effect of chemotherapy. Cancer Res 2002, 62:5476-5484

13. Bergers G, Song S, Meyer-Morse N, Berglund E, Handhan D: Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest 2003, 111:1287-1295

14. Löfblom J, Feldwisch J, Tolmachev V, Carlsson J, Ståhl S, Frejd FY: Affibodymolecules: Engineered proteins for therapeutic, diagnostic and biotechnological applications. FEBS Letters 2010, 2670-2680

15. Nygren P-Å: Alternative binding proteins: Affibody binding proteins developed from a small three-helix bundle scaffold. FEBS J 2008, vol 275:2668-2676

16. Feldwish J, Tolmachev V, Lendel C, Herne N, Sjöberg A, Larsson B, Rosik D, Lindqvist E, Fant G, Höidén-Guthenberg I, Galli J, Jonasson P, Abrahmsén L: Design of an Optimized Scaffold for Affibody molecules. J Mol Biol 2010, 398, 232-247

17. Ahlgren S, Orlova A, Wållberg H, Hansson M, Sandström M, Lewsley R, Wennborg A, Abrahmsén L, Tolmachev V, Feldwisch J: Targeting of HER2-expressing tumors using ^{111}In-ABY-025, a second-generation Affibody molecule with fundamentally reengineered scaffold. J Nucl Med 2010, 51:1131-1138

18. Andersen JT, Pehrson R, Tolmachev V, Daba MB, Abrahmsén L, Ekblad C: Extending half-life by indirect targeting of the neonatal Fc receptor (FcRn) using a minimal albumin binding domain. J Biol Chem 2011, Vol286, NO.7; 5234-5241

19. Tolmachev V, Orlova A, Pehrson R, Galli J, Baastrup B, Andersson K, Sandström M, Rosik D, Carlsson J, Lundqvist H, Wennborg A, Nilsson FY: Radionuclide Therapy of HER2-Positive Miroxenografts Using a ^{177}Lu-labeled HER2-Specific Affibody Molecule. Cancer Res 2007, 67(6): 2773-82

20. Loskog A, Dzojic H, Vikman S, Ninalga C, Essand M, Korsgren O, Totterman T H: Adenovirus CD40 ligand gene therapy counteracts immune escape mechanisms in the tumor microenvironment. J Immunol 2004, 172; 7200-7205

21. Frey AB, Monu N: Signaling defects in anti-tumor T cells. Immunol rev 2008, Vol. 2222: 192-205

22. Ochsenbein AF, Klenerman P, Karrer U, Ludewig B, Pericin M, Hengartner H, Zinkernagel RM: Immune surveillance against a solid tumor fails because of immunological ignorance. Proc Natl Acad Sci USA 1999, Vol. 96:2233-2238

23. Hank JA, Surfus JE, Gan J, Jaeger P, Gillies SD, Reisfeld RA, Sondel PM: Activation of Human Effector Cells by a Tumor Reactive Recombinant Anti-

Ganglioside GD_2 Interleukin-2 Fusion Protein (ch14.18-IL2). Clin Cancer Res 1996, Vol2. 1951-1959

24. Heuser C, Ganser M, Hombach A, Brand H, Denton G, Hanisch F-G, Abken H: An anti-MUCI-antibody-interleukin-2 fusion protein that activates resting NK cells to lysis of MUCI-positive tumour cells. British J Cancer 2003, 89, 1130-1139

25. Malek TR: The biology of interleukin-2. Annu Rev Immunol 2008, 26:453-79

26. Osenga KL, Hank JA, Albertini MR, Gan J, Sternberg AG, Eickhoff J, Seeger RC, Matthay KK, Reynolds CP, Twist C, Krailo M, Adamson PC, Reisfeld RA, Gillies SD, Sondel PM: A Phase 1 Clinical Trial of the hu14.18-IL2 (EMD273063) as a Treatment for Children with refractory of Recurrent Neuroblastoma and Melanoma: a Study of the Children's Oncology Group. Clin Cancer Res 2006, 12(6)

27. Carnemolla B, Borsi L, Balza E, Castellani P, Meazza R, Bernt A, Ferrini S, Kosmehl H, Neri D, Zardi L: Enhancement of antiumor properties of interleukin-2 by its targeted delivery to the tumor blood vessel extracellular matrix. Blood 2002, 99:1659-1665

28. Schliemann C, Palumbo A, Zuberbühler K, Villa A, Kaspar M, Trachsel E, Klapper W, Dietrich Menssen H, Neri D: Complete eradication of human B-cell lymphoma xenografts using rituximab in combination with the immunocytokine L19-IL2. Blood 2009, 113:2275-2283

29. Jahannsen M, Spitaleri G, Curigliano G, Roigas J, Weikert S, Kempkensteffen C, Roemer A, Kloeters C, Rogalla P, Pecher G, Miller K, Berndt A, Kosmehl H, Trachsel E, Kaspar M, Lovato V, González-Iglesias R, Giovannonoi L, Menssen H D, Neri D, de Braud F: The tumour-targeting human L19-IL2 immunocytokine: Preclinical safety studies, phase I clinical trial in patients with solid tumours and expansion into patients with advanced renal cell carcinoma. European J Cancer 2010, 46:2926-2935