

Breast cancer and HER3; the possibilities of imaging with VHH probes

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5/9/2011 - 18/11/2011

Abstract

Breast cancer is one of the most common cancers in the world and HER2-amplified breast cancer represents about a quarter of all breast cancer cases. HER2-amplified breast cancer is an aggressive form of breast cancer. In order to be able to diagnose this type of cancer in an early stadium and to find a specific treatment, research has been done on possible targets. Studies have pointed out that HER3 plays an important role in HER2-amplified breast cancer and in breast cancer cases where endocrine resistance originated and that this might be a target for imaging and possible treatment. The use of Variable heavy chain of heavy-chain-only antibody (VHH) in optical imaging can simplify and improve the quality of imaging and treating breast cancer tumors. The images can be obtained faster and they might have better signal-to-background ratios than ones made with conventional antibodies, due to their particular features (such as size, stability, and specificity).

In this research we have investigated a number of VHHs that in the near future could be useful for HER3 imaging in breast cancer tumors. The VHHs tested did not activate the downstream pathway of HER3 and did not seem to compete with its ligand, neuregulin (except for E11 VHH). E11 showed binding to HER3 on the cell surface (being conjugated to a fluorophore) as well as with only the extracellular domain of HER3. Although the wanted high affinity was not found in this study, imaging with near infrared and ELISA showed that E11 had the best results from the tested VHHs. These results implicate that E11 possesses some of the properties wanted for a probe for imaging, but that the affinity appears to be the limiting factor for the imaging of HER3 in breast cancer. Future research might point out a VHH that is able to bind with the wanted high affinity.

Keywords: HER2-amplified breast cancer, VHH, near infrared fluorophore imaging, HER3, breast cancer

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Introduction

This thesis begins with the preliminary literature research for the practical research. Information on breast cancer is given in chapter 1. The epidermal growth factor receptor family contributes to various processes in the cell that are highly active in cancer cells. Basic information on these receptor tyrosine kinases is given in chapter 3 and the role of HER3 in HER2-amplified breast cancer is pointed out in chapter 4. The major role of HER3 in HER2-amplified breast cancer provides a possible target for imaging and therapy. The type of imaging that could be used for HER3 imaging in breast cancer tumors, that is not invasive and is relatively easy to use, is optical molecular imaging, namely near infrared fluorescence imaging (chapter 2). This non-invasive type of imaging in combination with the use of VHHs, can result in faster, cheaper and better imaging than in combination with monoclonal antibodies. How these VHHs look like, work and are being produced is shown in chapter 5. This literature research provides information on the higher purposes of the practical research, which is described in chapter 6. In this chapter a summary is given on the hypothesis/questions that were relevant for the planning and execution of the practical research. The ultimate goal of the practical work was to find a convenient VHH to use for HER3 imaging of breast cancer tumors. Finally, in chapter 7 suggestions are given for the continuation of this research, towards the development of VHH probes for optical imaging of HER3.

Chapter 1: Breast Cancer

1.1: Place of origin and stages

Breast cancer is a type of cancer which develops in the breast tissue of women or men. In figure 1 the anatomy of the female breast is shown. As can be seen, the breast mainly exists of lobules (or glands) and fat. In case of breast cancer, there is abnormal growth of the cells in the glands or in the ducts that connect the glands to the nipple. A breast cancer can be defined as malignant when cancer cells invade the surrounding tissue. Breast cancer is inherited in 5-10% of all cases, in the other 90% it is due to an error in the persons genetics that occurred during lifetime. Since this form of cancer occurs mostly in women, the focus will be on breast cancer in women.

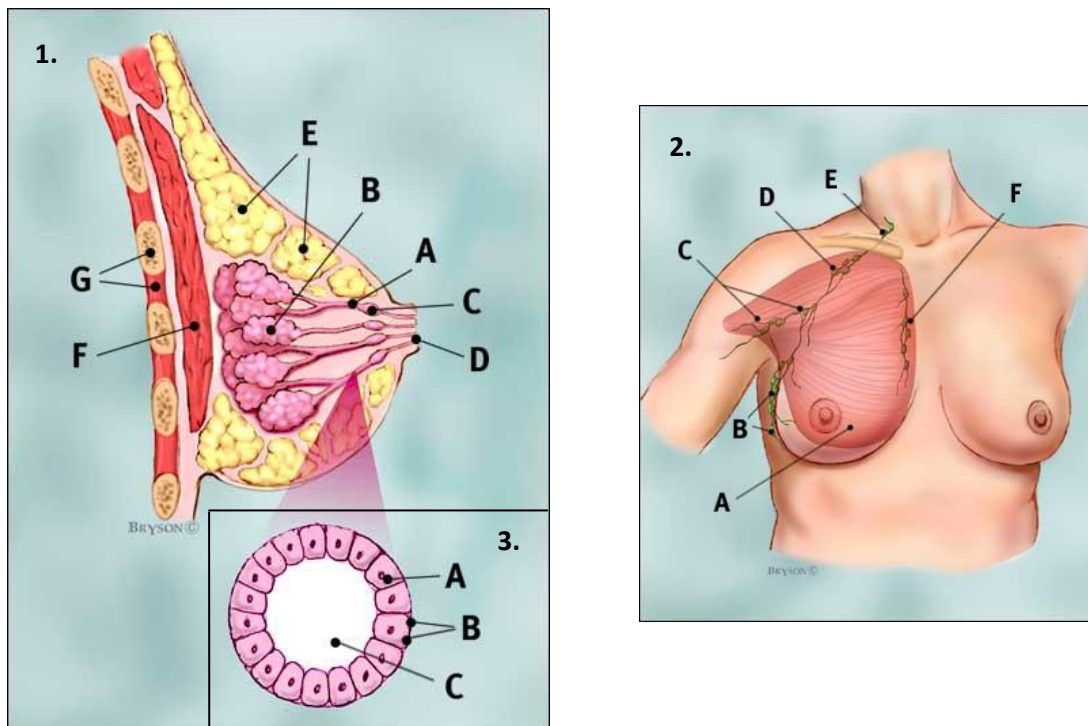


Figure 1: The anatomy of the female breast (2).

- | | | |
|---|---|--|
| <p>1.
 A: ducts
 B: lobules
 C: dilated section of duct to hold milk
 D: nipple
 E: fat
 F: pectoralis major muscle
 G: chest wall/rib cage</p> | <p>2.
 A: pectoralis major muscle
 B: axillary lymph nodes: levels I
 C: axillary lymph nodes: levels II
 D: axillary lymph nodes: levels III
 E: supraclavicular lymph nodes
 F: internal mammary lymph nodes</p> | <p>3.
 A: normal duct cells
 B: basement membrane
 C: lumen (center of duct)</p> |
|---|---|--|

There are different types of breast cancer, based on the tissue the cancer initiates from and how it evolves (1).

- Ductal carcinoma in situ
- Invasive ductal carcinoma
- Invasive ductal carcinoma (less common types)
- Invasive lobular carcinoma
- Inflammatory breast cancer
- Lobular carcinoma in situ
- Male breast cancer
- Paget's disease of the nipple
- Phyllodes tumors of the breast
- Recurrent and metastatic breast cancer

The breast cancer develops following a number of different stages, the different stages can be found in table 1. In these stages the cancer can develop from a single cell mutation to a cancer that has spread out to and through the lymph nodes.

Stage	Definition
Stage 0	Cancer cells remain inside the breast duct, without invasion into normal adjacent breast tissue.
Stage I	Cancer is 2 centimeters or less and is confined to the breast (lymph nodes are clear).
Stage IIA	No tumor can be found in the breast, but cancer cells are found in the axillary lymph nodes (the lymph nodes under the arm) OR the tumor measures 2 centimeters or smaller and has spread to the axillary lymph nodes OR the tumor is larger than 2 but no larger than 5 centimeters and has not spread to the axillary lymph nodes.
Stage IIB	The tumor is larger than 2 but no larger than 5 centimeters and has spread to the axillary lymph nodes OR the tumor is larger than 5 centimeters but has not spread to the axillary lymph nodes.
Stage IIIA	No tumor is found in the breast. Cancer is found in axillary lymph nodes that are sticking together or to other structures, or cancer may be found in lymph nodes near the breastbone OR the tumor is of any size. Cancer has spread to the axillary lymph nodes, which are sticking together or to other structures, or cancer may be found in lymph nodes near the breastbone.
Stage IIIB	The tumor may be of any size and has spread to the chest wall and/or skin of the breast AND may have spread to axillary lymph nodes that are clumped together or sticking to other structures, or cancer may have spread to lymph nodes near the breastbone. Inflammatory breast cancer is considered at least stage IIIB.
Stage IIIC	There may either be no sign of cancer in the breast or a tumor may be any size and may have spread to the chest wall and/or the skin of the breast AND the cancer has spread to lymph nodes either above or below the collarbone AND the cancer may have spread to axillary lymph nodes or to lymph nodes near the breastbone.
Stage IV	The cancer has spread — or metastasized — to other parts of the body.

Table 1: the different stages of breast cancer (2).

1.2: Symptoms of breast cancer

In the beginning, the symptoms of a breast cancer can be just a small lump. The texture of this differs, but mostly it is a hard uneven lump. The list below shows other symptoms that can be caused in the breast by the cancer, according to the American Cancer Society.

- swelling of all or part of the breast
- skin irritation or dimpling
- breast pain
- nipple pain or the nipple turning inward
- a nipple discharge other than breast milk
- a lump in the underarm area
- redness, scaliness, or thickening of the nipple or breast skin

1.3: Breast cancer diagnosis

The first step in detecting breast cancer can be literally “in the patient’s own hands”. By examining the breasts, possible lumps can be detected (3).

With an x-ray mammography, lumps that could indicate breast cancer can be observed. This breast cancer screening with X-rays is mostly done with women between the ages of 50 and 75. Because the breasts of younger women contain more fatty and connective tissue. This means that an early tumor can hardly be visualized (4).

When a lump is found in the breast tissue, this has to be examined taking a puncture or biopsy. With a cytological puncture, liquid is taken from the tumor that contains tumor cells, with a thin needle. These cells are then examined with a microscope within a few hours. In the case of a biopsy, a piece of the suspected tumor is taken with a thick needle or is being cut out. This technique requires more time to obtain the results, but these results are more trustable (5).

To define the type of breast cancer, pathological research can be done. This is crucial in finding the appropriate therapy and this information can be used for future cases.

Two major causes of breast cancer can be either a defect in HER2 or in a hormone receptor. If the cancer is caused by a hypersensitive hormone receptor, this can cause the abnormal cell division and proliferation, and therefore the therapy should interact with those receptors. Overexpression of HER2 is another possibility that can be found in about 20% of all breast cancer cases.

Two other types of analysis can be done to create a better idea of the cause of breast cancer. By looking at biomarkers in the body (most of the times in the bloodstream) that might be related to inheritable breast cancer; or by genomic research, which can point out possible mutations that can cause inheritable breast cancer. These results can be used to create gene-profiles, information about a tumor that can be used to define tumors that will be found in the future (6).

1.4: Breast cancer therapies

The treatment of breast cancer depends on different aspects of the cancer and of the patient:

- Stage and grade of the cancer
- Patients age
- Pre/post menopause
- Size of the tumor
- overexpression of hormone/protein receptors

Surgery

With primary breast cancer, i.e. cancer localized only in the breast , the tumor will be removed by surgery. If necessary the surgeon can also remove the lymph node(s) close to the tumor. Afterwards, the breast tissue is treated with radiotherapy to make sure all the cancer cells are destroyed. When the tumor is large, it can be necessary to shrink the tumor with chemotherapy or hormonal therapy (neo-adjuvant) before it can be removed surgically (7).

Chemotherapy

This particular treatment is applied in case of the two following situations. The first one is as described above, to kill any remaining cancer cells after surgery or to shrink the tumor before the surgery. The second use is when the cancer has developed too much to be removed surgically, and has metastasized. The chemotherapy is then used to “control” the cancer as much as possible (8).

Radiation therapy

This therapy uses radiation to kill cancer cells. The therapy reacts more to cancer cells than the normal cells, because the radiation interacts mostly with two processes: cell growth and division . Furthermore the defense mechanisms in cancer cells are much weaker and the repair mechanisms are not as good as in normal body cells. This means that the damage done by the radiation is more permanent and prevents the cancer cells from living (9).

Hormonal therapy

If cancer cells overexpress a hormone-receptor, hormonal therapy can be used. Of all breast cancer cases 80% is estrogen-receptor-positive, this means that the estrogen receptor is overexpressed by the cancer cells. This means that this therapy is only effective in breast cancer cases that overexpress these hormone receptors. This type of therapy either lowers the amount of a hormone (estrogen or progesterone) or blocks action of the hormone. These two mechanisms can result in a decrease in chance that the cancer will come back after surgery or it can help to slow down (hormone-receptor-positive) breast cancer in advanced stages. It is also used with women with a high risk factor for getting hormone-receptor-positive breast cancer, before it is diagnosed. In some cases of hormone-receptor-positive breast cancer with pre-menopausal women the ovaries (major producers of estrogen) are shutdown by medication or by surgically removing them in addition (10).

Targeted therapies

With this treatment there are specific aspects of cancer cells that the treatment aims for. This means that the normal cells around the tumor are not damaged. Treatments used for breast cancer nowadays are Herceptin and Tykerb, they work both on HER2, and Avastin. Avastin is an angiogenesis inhibitor (11).

Chapter 2: Molecular imaging

Molecular imaging can be defined as “the visualization, characterization, and measurement of biological processes at the molecular and cellular levels in humans and other living systems”(12).

There are many technologies for molecular imaging, for example: magnetic resonance, positron emission tomography and optical imaging. Since the practical research in this thesis is on optical imaging of HER3 the focus of this chapter will be on optical imaging, especially employing near infrared (NIR) fluorophores (13).

Optical imaging is a non-invasive technique that can be applied for medical uses, for example for screening for breast cancer. In 1929 M. Cutler was the first to use optical imaging for breast cancer detection. He used translumination to detect lesions in breasts. This means that the breast was examined in a complete dark room with a device that emitted light behind the breast. The different levels of absorption of the light could indicate the different tissues in the breast. Since this simple technique, a significant progress has been made in terms of sensitivity and specificity.

There are three different types of optical imaging nowadays. The first technique makes use of only the difference in absorption of the different wavelengths a breast is exposed to, due to different optical properties of the tissue components (hemoglobin, water, lipids). The other techniques use a contrast agent: either a targeted or a non-targeted agent. Contrast agents are chemical substances that are excited by a certain wavelength and, during their decay, emit fluorescence of a particular wavelength. (14). The tissue exposed to the near infrared spectrum absorbs the energy from the light. This absorption leads to an elevation of the dye from its steady state to a high-energy state. When the dye returns to its normal steady state it emits energy. This energy exists of photons, for example in the NIR range. The photons emitted by this probe, are then detected and used to make an image. The excitation light is of a different wavelength than the photons that are emitted, so that it can be filtered out and only the photons can be detected. Targeted agents are considered to be advantageous to non-targeted, as their specific association with particular tumor markers can give more information to the clinicians. These agents need to have high specificity for their targets and these targets should be only present on tumor cells, to prevent high background signals. An example for additional specificity is when a NIR pH-activatable probe is used as a targeted contrast agent. This probe can be used in vivo and is activated by the different pH of the tumor, compared to normal tissues. The background signal of the image that is then obtained is low and the image provides a specific signal(15). Monoclonal antibodies have been used for purposes of targeted-contrast agents, however there are also some disadvantages of using monoclonal antibodies (namely long half-life in the bloodstream and poor tumor penetration), and smaller antibody fragments or single domain antibodies (namely VHHs) might be a favorable new alternative, more on this topic will be discussed in chapter 5.

Contrast agents can be (or be composed of) fluorophores that emit light in the near infrared region of the spectrum, between the wavelengths of 650-900 nm. This imaging window excludes wavelengths below 600 nm at which hemoglobin has a strong absorption and tissue auto-fluorescence is known to be more pronounced. Moreover, this imaging window excludes wavelengths above 900 nm at which water and lipids absorb light. Imaging with probes using the NIR spectrum does not only avoid the disadvantages of using probes that use visible light (such as a relatively low signal-to-background ratios and high autofluorescence), they also have deeper penetration (up to 15 cm) through the tissues (because of less auto-fluorescence and less absorption by tissue components) (16). The emitted NIR light is not visible for the human eye, special machinery is needed to make it visible(13). These imaging machines detect the light absorbed by tissue or the light that is scattered by tissue (17). The emitted photon travels through the tissue.

The directions of the photons depend on the tissue's scatter, anisotropy and refractive index. Scatter indicates the amount of signal that did not come straight from its source without having diverged from its direct linear path and so creating a misleading image. Anisotropy is when the features of the tissue are depending on the direction and angle that the photon is traveling in and the refractive index indicates the amount of refraction when the photon enters different materials/tissues (18).

Chapter 3: The human epidermal growth factor receptor family

The human epidermal growth factor receptor family is a group of receptor tyrosine kinases, consisting of four different types (19). They participate in several important mechanisms in the cell; proliferation, differentiation, adhesion, survival and migration. The receptors can transfer a signal from outside the cell, over the membrane, into the cell, and some of them need to form dimers to do this. All of the receptors in this family possess two cysteine regions in their extracellular domain (20).

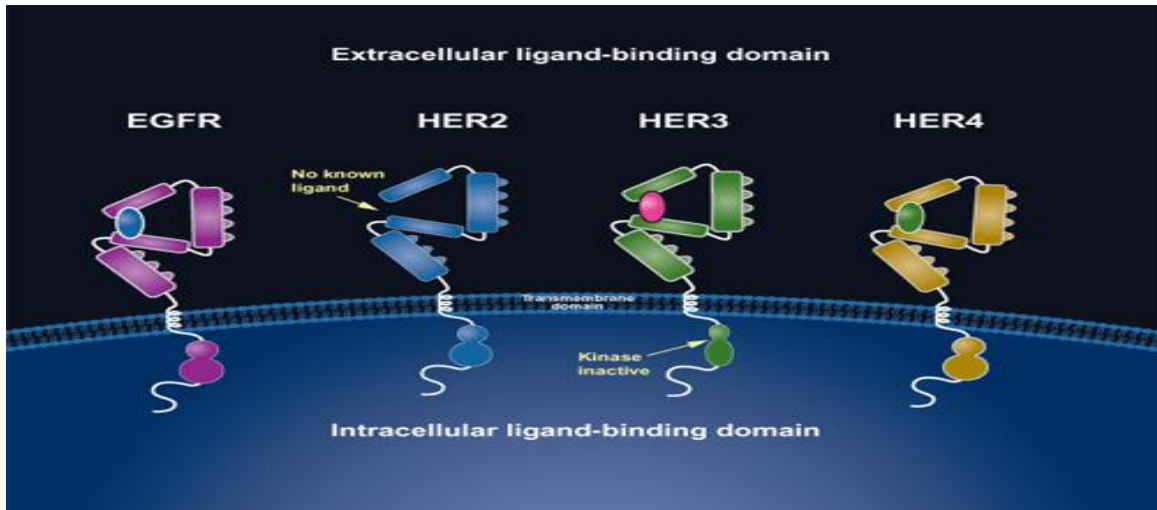
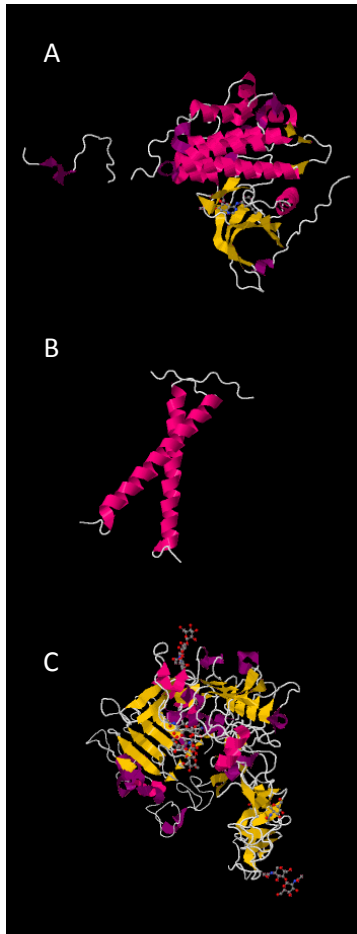


Figure 2: the four different members of the Her family (19).

HER1 (EGFR or c-erbB-1)



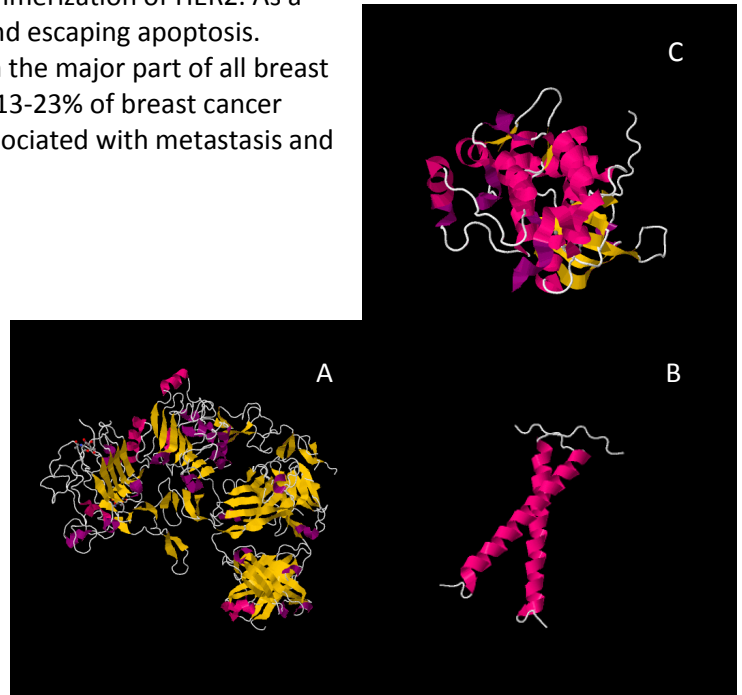
The gene for HER1 is localized on chromosome 7p12.3-p12.2. HER1 weighs 170 kDa and it is composed by an external and an internal domain connected to each other by a transmembrane domain (21). The external domain exists of 622 amino acids and 12 possible sites for N-linked glycosylation (22). This domain consists of four sub-domains, which are organized in a closed or an open conformation. In the closed conformation the receptor is not able to interact with its ligands. Some of the ligands of HER1 are: epidermal growth factor, transforming growth factor-alpha, androgen, heparin-binding EGF-like growth factor, betacellulin, amphiregulin and epiregulin (20). A high level of expression of these ligands is present in 85% of all breast cancer cases. This high level of expression appears to be independent of HER2 overexpression. The binding domain for the epidermal growth factor is situated between two cysteine-rich domains. The transmembrane domain contains 23 amino acids and the internal domain 542 amino acids (22). HER1 is involved in the development, differentiation and growth of several organs (20). The amount of receptors on the cell surface can be higher in cancer cells, this is the case in 2.7% of the breast cancer cases with a poor prognosis. The overexpression of HER1 is more associated with one particular type of breast cancer, basal-like breast carcinoma, in 54% of the cases.

Figure 3: A) The extracellular domain of EGFR. B) The transmembrane domain of EGFR. C) The intracellular domain of EGFR (21).

HER2 (neu or c-erbB-2)

The gene of HER2 is localized on chromosome 17q21, the produced protein weighs 185 kDa. HER2 possesses only intracellular kinase activity, this means that there is no activation by a ligand. It is the family member that has the best ways to interact with the others. A mutation that can occur on the HER2 gene (amino acid codon 655) causes a change in the structure (23). This structure change activates the transmembrane domain, this is a signal to form homodimers. This mutation or overexpression of HER2 can result in the homodimerization of HER2. As a result the (cancer) cell will grow uncontrolled and escaping apoptosis. Several studies point out that HER2 is present in the major part of all breast cancer cases and that overexpression occurs in 13-23% of breast cancer cases(24, 25). The overexpression of HER2 is associated with metastasis and an aggressive phenotype (20).

Figure 4: A) The extracellular domain of HER2. B) The transmembrane domain of HER2. C) The extracellular domain of HER2 with Herceptin Fab (21).



HER3 (c-erbB-3)

The HER3 gene is localized on chromosome 12q13 and the receptor weighs 145 kDa. It possesses a ligand binding site that interacts with neuregulin1&2 and intracellular kinase activity. HER3 plays a major role in the development of the neural system, it controls the growth of Schwann cells. HER3 deficient mice appeared not to survive the embryonic stage or die not long after being born (26). Mutations in this receptor make it unable to transfer signals via a downstream pathway without dimerization. In a breast tumor overexpressed HER3 promotes an anti-apoptotic reaction (20). This overexpression occurs strongly in 17.5 % of all breast cancer cases (25). HER3 can produce an anti-apoptotic reaction, this is in the case of cancer beneficial for the tumor growth. HER3 often forms heterodimers with HER2. These hetero dimmers appear to be a crucial target in possible therapies for breast cancer. This will be discussed in chapter 4, the chapter dealing with the relationship between HER3 and breast cancer.

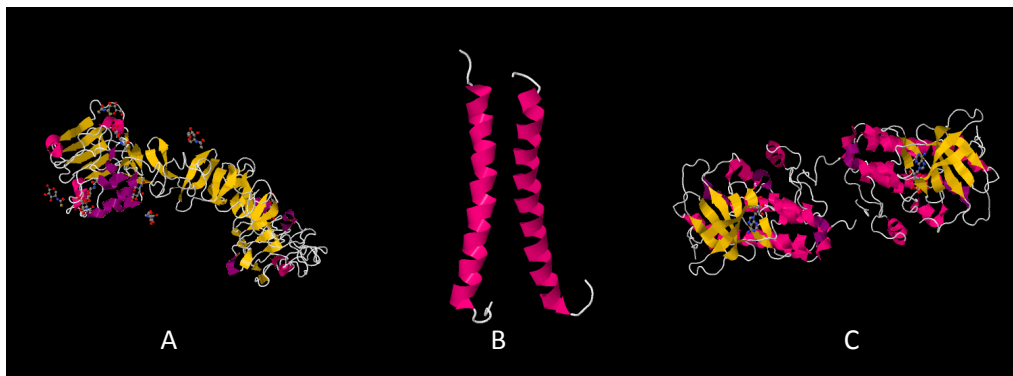


Figure 5: A) The extracellular domain of HER3. B) The transmembrane domain of HER3. C) The intracellular domain of HER3 (21).

HER4 (c-erbB-4)

HER4 was classified in 1993 and its gene is localized on chromosome 2q33 and the receptor weighs 180kDa. HER4 is a member of the family that does not require dimerization to activate the downstream pathways. It interacts extracellular with all the four subtypes of neuregulin heparin-binding EGF-like growth factor and epiregulin. HER4 expression occurs in 11.9% of breast cancers, but it is related to downgrading the cell proliferation (25). Therefore this (over)expression of the HER4 protein is associated with a treatable form of breast cancer. The prognosis with HER4 gene expression however is worse (20).

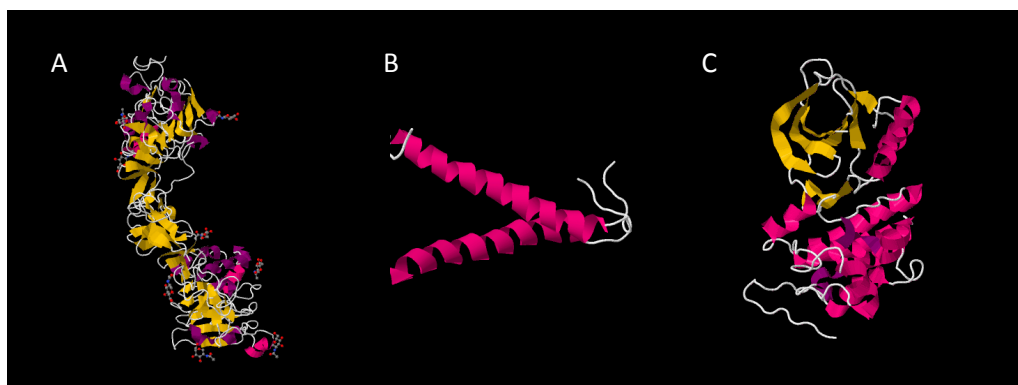


Figure 6: A) The extracellular domain of HER4. B) The transmembrane domain of HER4. C) The intracellular domain of HER4 (21).

Chapter 4: Breast cancer and HER3

As stated earlier, HER2-amplified breast cancer covers about a third of all breast cancer cases. In 20-30% of the breast cancer cases there is HER2 overexpression (27). Although HER2 has no described ligand, it is in its open conformation and therefore easily forms homodimers or/and heterodimers, becoming constitutively active. Treatments that target HER2 trigger multiple reactions in order to block this downstream pathway (of the dimers). Herceptin diminishes the downstream pathway of HER2 by internalizing or degrading HER2. Moreover it can inhibit angiogenesis, HER2 extracellular domain cleavage and DNA repair. Besides acting as an inhibitor Herceptin can induce apoptosis, cell cycle arrest in G1 and attract other cells of the immune system (28). But these dimers could also consist of HER2-HER3 or HER2-EGFR, indication that there could be other targets than HER2 to aim for in HER2-amplified breast cancer. Studies have shown that blocking EGFR does not affect the amount of cell proliferation. Cell proliferation is a process in which HER2 is normally involved. The effect of a HER3 knockdown produced roughly the same effect as a HER2 knockdown *in vitro* in cell lines with HER2 overexpression, when looking at the cell division. In figure 7 this is illustrated with a test with knockdowns of HER1/HER2/HER3 in HER2 overexpressing cell lines (29). HER3 shows in *in vivo* like conditions to effect the 3d growth. The knockdown of HER3 inhibits the growth. *In vivo* the knockdown of HER3 even shows tumor regression (29).

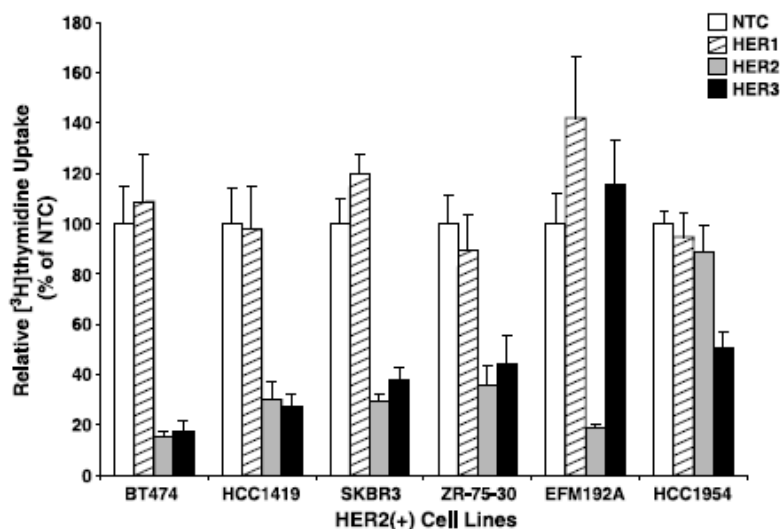


Figure 7: The effect of HER1, HER2, HER3 knockdown on HER2 overexpressing cell lines. HER1 (EGFR) hardly affects the proliferation measured by [³H]thymidine uptake. The knockdown of HER2 and HER3 both result in the same decrease of cell division in all but one cell line (29).

The fact that several studies point out that HER3 is a major factor in HER2-amplified breast cancer, creates new possibilities. HER3 could also be used as a target for therapy for HER2-amplified breast cancer. The treatment now used for HER2-amplified breast cancer is targeted for HER2 (trastuzumab) and this treatment does not work in all cases. Moreover, patients can create a resistance for the therapy. A treatment targeting HER3 might avoid the problems that occur with the HER2 treatment (30). Moreover, recent data has implicated HER3 activity in tamoxifen-resistant and fulvestrant-resistant breast cancer cell. In HER2-amplified breast cancer cell lines, when treated with fulvestrant, an unchanged level of HER3 and in breast cancer cell lines with low levels of HER2 a upregulation of Her3 was found. This is a unwanted side effect besides the growth-inhibitory effect achieved by fulvestrant in ER-positive breast cancer cells (31).

For these reasons, molecular imaging of HER3 is becoming very relevant and the development of probes for HER3 essential. These probes need to be able to bind to their target specifically, like antibodies do. Creating probes based on antibodies could lead to fast imaging with high contrast images.

Chapter 5: Variable heavy chain of heavy-chain-only antibodies

Monoclonal antibodies (MAbs) have been developed for therapies (e.g. trastuzumab which is anti-HER2 therapy) and their specificity has been used for molecular imaging techniques. The process of creating these molecules is very complex and time consuming, making the monoclonal antibodies very expensive. Due to the relative large size of the MAbs they tend to react in a negative way on unusual temperatures and pH values. Their size also limits the reach of the MAbs, for example they cannot reach throughout a whole solid tumor (poor penetration, slow diffusion). They are also cleared very slowly, which can be an advantage for some therapeutic applications, but a disadvantage for imaging applications. This is where the camelid single-domain antibody (sdAb), VHH (variable domain of the heavy chain of heavy-chain-only antibody) or nanobodies could make the difference (32).

5.1: Structure of VHHs

In *camelidae* the first step towards nanobody (or VHH) technology was discovered (33). The research group of Prof. R. Hamers was the first to see the different structure of the *camelidae* antibodies. Besides the conventional antibodies, these animals also possess heavy chain-only antibodies that only consist of a single variable domain connected to two constant domains with a hinge (figure 8). The two constant domains (CH2 and CH3) of the heavy chain antibody are similar to the Fc domains of the conventional antibodies. The conventional antibodies, however, contain one component more in their constant domain (34). The variable domain is capable of antigen binding in the same way as a normal heavy-chain antibody would do. The VHHs are very stable and soluble. They weigh only 15kDa, although they are still fully functional. This makes them the smallest, non-synthetic molecules, that can bind an antigen without the use of a light chain (35).

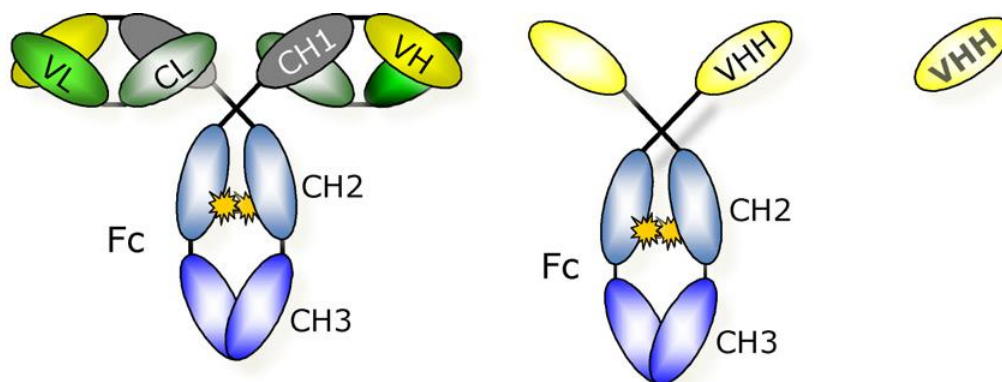


Figure 8: From left to right: a conventional antibody, a camelidae antibody and a VHH or nanobody (33).

The scaffolds of the VHH are made of two β -sheeted parts. This is very similar to the variable heavy chain structure in a conventional antibody, but the antigen-binding loops are different on the VHH. The VH and VHH are both built up from four conserved sequence parts, the framework regions and the complementarity determining regions (CDRs) (33). These antigen binding loops of the VHHs appear to have more possible binding options than the conventional antibodies. The CDR regions are roughly in the same place, but the CDR1 and CDR3 regions are different from the VHH (figure 9). CDR1 of VHH is located more towards the N terminus and CDR3 from a human VH is 12 amino acids and in dromedary-derived VHHs the length is between 16-18 amino acids (36). The sequences of amino acids of these VHHs also seem to be different from what is found in the VH parts of antibodies that do contain a light chain. This looks like the VHHs have evolved in a way to make up for the lacking light chain (35).

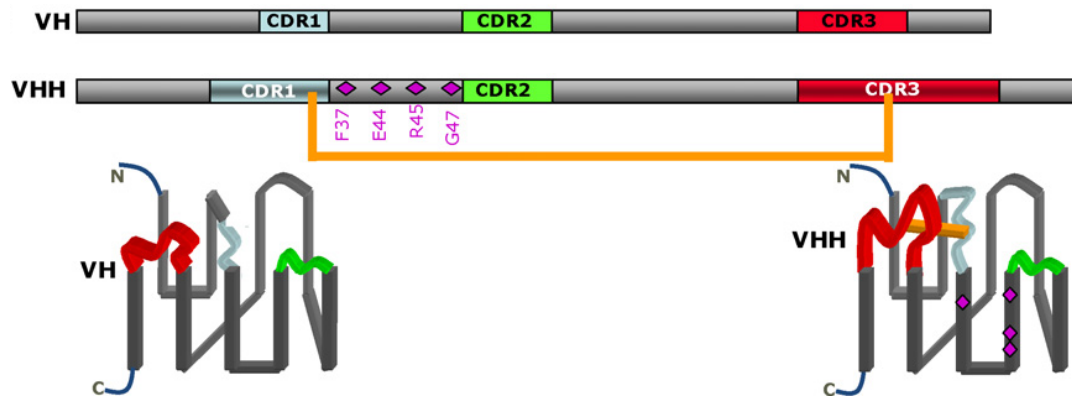


Figure 9: The similarities and differences between VH & VHH. The structure is a like, but the CDR1 and CDR3 of VHH are bigger and sometimes connected to each other with a disulfide bond (33).

5.2: Features of VHHs

A VHH combines the features of conventional antibodies with its own features making them interesting tools to develop for imaging or therapeutic applications. By avoiding problems such as a costly and time consuming production, instability that occurs due to their size, VHHs are very interesting compared to MAbs. Features that the VHHs have in common with the conventional antibodies are (37):

- High target specificity
- High affinity for their target
- Low inherent toxicity

Importantly, as stated before, they have certain advantages compared to the conventional antibodies. They are more stable, are easier to produce and there are other ways to administer them besides intravenous injection. VHHs are stable in the different conditions of pH and temperatures (37). Their clearance is, unlike that of MAbs, only a matter of hours (32). For imaging a fast clearance is preferred, but for some therapeutic purposes a longer exposure is needed.

5.3: Production of VHHs

VHHs can be produced using bacteria, yeast or fungi, instead of mammalian cell lines that are necessary for the production of MAbs. The production therefore is much faster, easier and cheaper (32). The production of VHHs is shown in figure 10. From an immunized *camelidae* the peripheral blood lymphocytes are used to make cDNA. All the VHHs can be obtained from one set of primers, because they are all part of the same gene family. After this, there is a second polymerase chain reaction (PCR), to create more material and to include restriction enzyme sites, which is necessary for cloning. A library is then created of VHHs containing all the antigen-binding sites that the heavy chain only antibodies of the *camelidae* had. From such a library, the VHHs can be selected based on their antigen-specific-binders. This can be done by either direct colony screening or by panning. Once selected, the VHHs can be produced in bacteria or lower eukaryotes. There are also other libraries that can be created, synthetic or naïve libraries. These seem to deliver VHHs with a much lower affinity and their affinity has to be improved by “maturing” the antibody fragments. These alternatives are more time consuming than the method with a library made from an immunized animal (35). VHHs can be linked together to form bivalent proteins that can either bind two antigen parts close to each other (biparatopic) or two different antigens (bispecific) . This could raise the avidity whilst containing the fully functional binding domains.

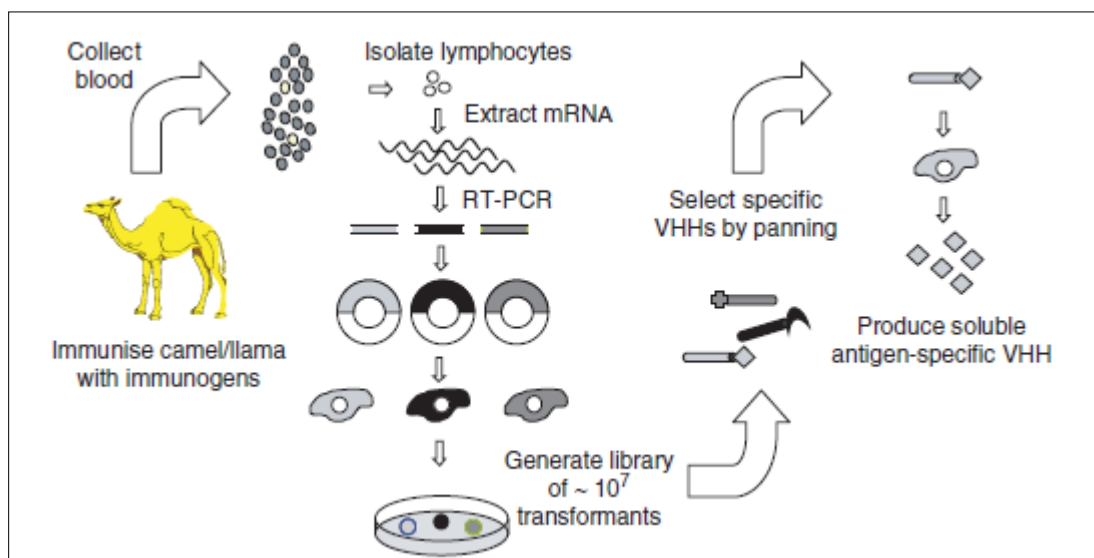


Figure 10: The production of VHHs from a *camelidae* (33).

5.4: Use of VHHs

All the characteristics of the VHHs make them a good candidate to be used for medical purposes. They can be used in various treatments, for example VHHs can be used to inhibit EGF-dependent cell proliferation. This has been accomplished by blocking the binding of EGF to its receptor and thereby inhibiting the downstream pathway. This is a signal that often occurs constitutively with solid tumors (38). Another example of medical use of VHHs is for rheumatoid arthritis treatments, for which they are used to block TNF α (tumor necrosis factor alfa). TNF α is a cytokine that plays a major role in the damage caused in the joints when suffering from rheumatoid arthritis (39).

Besides the therapeutic applications, the VHHs can be useful in molecular imaging. This imaging can be used as a tool to help clinicians with screening for tumors, with a diagnosis or to see if a patient is responding to therapy. Such an image can be analyzed, paying attention to the target of the VHH. This can be a receptor that is overexpressed on the tumor cell surface, in this case it can be used to localize this tumor and see if there is a size difference compared to a previous image (34). A VHH makes use of its high affinity to create a precise signal with a high background ratio, this means that it binds mostly to its target and that only the target emits photons and not the surrounding tissue. Because the VHH is small it can diffuse rapidly and penetrate tumors easily, it can reach throughout the whole tumor, where conventional antibodies cannot. Their size also makes it possible to provide an image very short after the administration of the VHH, because they reach their target fast and rapidly penetrate the tumor, and are rapidly cleared from normal tissues and from the bloodstream (35).

Apart from these medical applications, the VHHs can also be used in various biotechnological applications. In living cells, VHHs can visualize antigens, when a VHH is linked to for example a fluorophore group. This type of imaging was discussed in the chapter on optical imaging. Another way to use VHHs for such an application is with anti VHH antibodies and secondary antibodies for imaging. This means that with this technique use of conventional antibodies is required (34).

Chapter 6: Practical Research

6.1: Introduction

As mentioned in this thesis, breast cancer is one of the most common forms of cancer and HER2 amplification occurs in 20-30% of all breast cancer cases. With an increasing number of evidences pointing at (overexpression of) HER3 having an important role in this form of breast cancer, as well as in the development of resistance to certain breast cancer therapies, it would be very useful to be able to image cells with (an overexpression of) HER3. The techniques that are commonly used make use of radioactive tracers, but optical imaging would be a faster way to detect without using radiation. The techniques used now for optical molecular imaging in such cases with monoclonal antibodies are very expensive and are not working optimally. Working with nanobodies (VHHs) could be used to image these cells faster, cheaper and more precise. This could result in earlier diagnosis of cancer, being able to see if a patient reacts to treatment or in the future even to new therapeutic applications. This research aims to investigate selected VHHs from previous studies that bind to the extracellular domain of HER3. These studies will determine:

- if the VHHs bind to cells expressing HER3 on their surface, compared with negative control cell line
- if binding of the VHHs induces activation of HER3, if it could affect the binding of neuregulin and the possible activation of downstream pathways.
- If the conjugation of a near-infrared fluorophore to the VHHs affects the binding.

6.2: Materials and Methods

Cell Lines and Culture Conditions

3T3 2.2 (murine fibroblasts), MCF7 (human breast cancer) and A431 (human vulva cancer) cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). 14C cell line (human head and neck cancer) was developed by Dr. T.E. Carey, Ann Arbor, MI). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with Fetal Bovine Serum (FBS) penicillin, streptomycin and glutamine at 37°C .

Nanobodies

The nanobodies used (i.e. E11, O6C6, and O7C4) were selected by Smiriti Raghoenath, based on studies performed with the extracellular domain of HER3. R2 was used as a negative control (40, 41).

HER3 detection in lysates of A431, MCF7, 14C and 3T3 2.2 cell lines by western blot

Cells were obtained from medium and counted, after that 100.000 cell per eppendorf were selected. The cells were pelleted by centrifugation (700 RCF for 1 minute), the supernatant was removed and the cells were washed with Phosphate Buffered Saline (PBS), after another centrifuge the reducing sample buffer (1x sample buffer, diluted with PBS) was added. The samples were then boiled and vortexed and afterwards put on the gel for protein separation according to molecular weight. Then, proteins were transferred to a nitrocellulose membrane by a semi-dry Western blot which was performed, 1 hour at 15 Volt followed by 0.5 hour with 25 Volt. The blocking was then done with 2% Bovine Serum Albumin (BSA) in Tris-Buffered Saline and Tween20 (TBST). The same solution was used to incubate the membrane. The membrane was incubated with the primary antibodies over night (a Rabbit anti-HER3, 1:1000 and a Mouse anti-actin 1:50.000). After the incubation the membrane was washed three times for five minutes in 1x TBST and then incubated for 1.5 hours with the secondary antibodies (Goat-anti-Rabbit 800nm and Goat-anti-Mouse 680nm, both 1:5000). After this incubation the membrane was washed the same way three times and an image was obtained and analyzed with Odyssey.

Activation and competition study with anti-HER3 VHH

Two days after 100.000 cells per well were seeded, the cells were serum starved over night (in DMEM with glutamine). The cells were then pre-incubated with anti-HER3 VHH at 4°C for 1 hour. The VHH used are: E11, O6C6, O7C4. R2 was previously used as a negative control in the binding study and therefore left out, this will be discussed later on. After the incubation with the VHHs the wells were incubated with neuregulin for another 15 minutes. The plates were then placed on ice and the medium was removed, after which the wells were washed with cold PBS. RIPA buffer was then added to the wells and the cells were scraped off and stored in eppendorfs on ice. Then sample buffer (4x sample buffer) was added and the samples were boiled for 5 minutes.

Two Western blots per cell line were performed and the blot was made with 1 hour at 15Volt and 0.5 hour at 25Volt. Per cell line there was one membrane incubated with Mouse-anti-actin and Rabbit-anti-HER3 and the other incubated with Mouse-anti-pAkt and Rabbit-anti-pHER3.

The membranes were then incubated with antibodies over night. After incubation the membranes were washed three times for 5 minutes with 10ml of 1x TBST. The membranes were then incubated for 1 hour with the secondary antibodies, in TBST 2% BSA, Goat-anti-Rabbit (800nm) and Goat-anti-Mouse (680nm). After the incubation the samples were washed three times with 1x TBST for 5 minutes again. The Odyssey scanner was used to image and analyze the membranes.

Binding assays:

The following different binding studies were performed:

1. binding of VHHs to 3 cell lines in presence or absence of neuregulin
2. binding of VHHs to MCF7 cells with higher concentration range
3. binding of E11, fresh O6C6 and O7C4 to MCF7 cells
4. binding of E11, fresh O6C6 and O7C4 to HER3 ECD
5. binding of IR-conjugated E11

For each binding assay (except mentioned otherwise) the following protocol was used:

Ten thousand cells per well were seeded 1 day before in 96-well plate. Cells were placed at 4°C for 5 minutes, and washed with DMEM buffer (Dulbecco's Modified Eagles Medium, 25mM Hepes and 1% BSA with pH 7.2). Then, cells were incubated with the VHH (E11, O6C6, O7C4, R2) in triplets for 1.5 hours at 4°C, to keep the VHHs from internalizing, on a rocker. The incubated cells were washed once with DMEM buffer and once with PBS and afterwards they were fixed with 4% paraformaldehyde (5 minutes at 4°C and 5 minutes at room temperature), so that the VHHs would not be internalized. The cells were then washed with PBS, PBS 1% BSA and incubated with the primary antibody, a Rabbit anti-VHH 1:1000 for 1 hour. The incubation of the secondary antibody (Goat anti Rabbit 800, 1:2000) for 45 minutes was done after, and also followed by two wash steps with PBS + 1% BSA. The amount of VHH bound to cells was detected and analyzed with the Odyssey scanner.

Binding assay 1 was performed with cell lines 3T3 2.2, 14C and MCF7, whereas all the other assays were only performed with MCF7 cells. The incubation with VHHs was performed with and without neuregulin (i.e. NRG), in equimolar conditions i.e. 50 nM concentrations.

Binding assay 2 was performed with higher concentration of VHHs, namely: 10nM, 50nM, 100nM, 500nM and 1000nM.

Binding assays 3 and 4 were performed with freshly made O6C6 and freshly made O7C4. The same protocol was followed as above, only there were 20.000 cells per well seeded for assay 2 and the ECD of HER3 was used in assay 4. For binding assay 3 and 4 concentrations of 500nM and 1000nM of VHH were used.

Binding assay 4 was performed with the extracellular domain (ECD) of HER3. The wells were coated with Fc-fusion HER3 ECD and incubated overnight at 4°C. The next day the wells were blocked with 4% Marvel in PBS for 30 minutes and afterwards the wells were washed three times. The wells were then incubated for 1 hour with the VHHs (E11, O6C6 old, O6C6 new, O7C4 old, and O7C4 new). Next, the wells were washed five times with 0.5% Tween20 in PBS and once with PBS and after the washing the wells were incubated for 1 hour with Rabbit anti-VHH (1:2500) followed by the same washing steps again. Then the wells were incubated with Donkey-anti-Rabbit-PO (1:3333). After the final washing steps, the substrate o-phenylenediamine (OPD) was added to the plates and when the wells turned bright yellow the reaction was stopped with sulfuric acid. The OD of the resulting stain was measured at 490 nm with a well plate reader.

Conjugation of IRDye800CW to VHH

Adding a fluorophore group to these VHHs creates the possibility to image the VHHs with optical imaging. To be able to see if the fluorophore group conjugated to a VHH, in this case IRDye800CW, influences the binding properties of this VHH a comparison was done between non-conjugated and conjugated VHH. Before that, and just after conjugation, the proteins were checked on gel for the successful conjugation or IRDye: The samples were made and boiled for 5 minutes and put on gel.

- 5.0 µl E11, 10.0 µl PBS and 5.0 µl 4x sample buffer
- 1.0 µl E11 + IRDye, 14.0 µl PBS and 5.0 µl 4x sample buffer

The gel was then stained with coomassie blue and the Odyssey used to image and analyze the gel.

Binding with IR-conjugated VHH (binding assay 5.)

Cells were collected and seeded again, according to the same procedure as before. Two 96-well plates were coated with gelatin and cells were added to the wells (10.000 cells per well). The cells of one plate were washed with DMEM after being cultured overnight. Triplets of cells with a dilution of conjugated E11 were added, the amounts are in nanomolar concentration (nM);

500	250	125	62,5	31,25	15,625	7,8125	3,90625	1,953125	0,976563
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The cells were incubated for 1.5 hours at 4°C and afterwards washed twice with DMEM. The 96-well plate was then imaged and analyzed with Odyssey.

6.3: Results

HER3 is clearly detected in MCF7 cell lysates and absent in 3T3.2 cell lysates

The membrane (figure 1) and gel (figure 2) were both scanned with the Odyssey scanner. The actin presence in all the different samples is approximately the same, so this means that about the same amount of cells is present in the different sample lanes. This means that the MCF7 cell line contains the most HER3, visible around 180 kDa according to this Western blot. The two other clear green bands, although not clear which proteins they represent, have also been documented in literature.

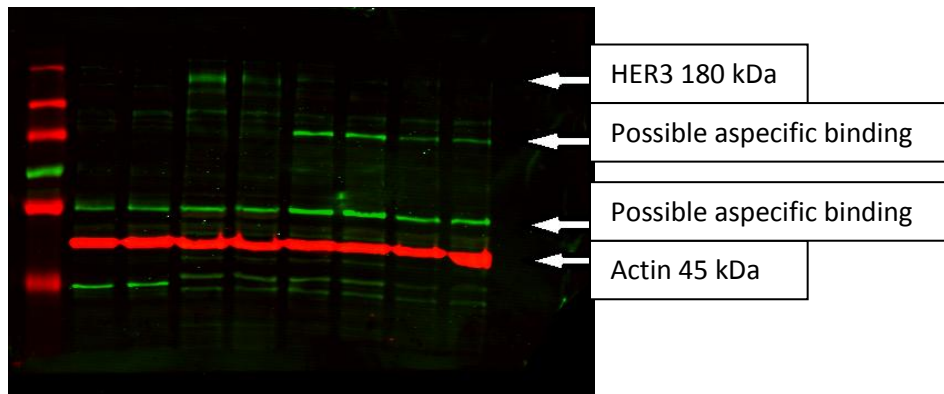


Figure 1: Odyssey scan with 800nm (green) and 700nm (red), with from left to right: ladder, 2x 3T3, 2x MCF7, 2x 14C and 2x A431.

In the scan of the gel are some bands left. This indicates that not all the proteins were transferred to the membrane from the gel.

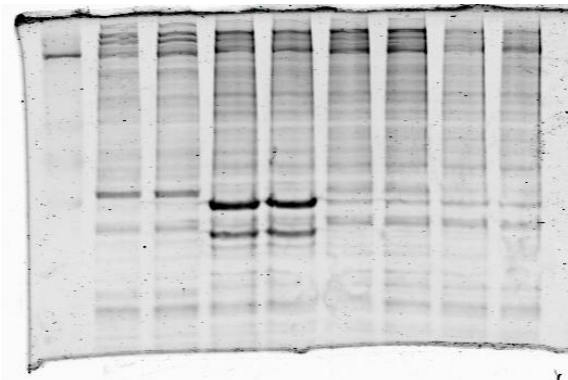


Figure 2: Gel after blot, 700i7, with from left to right: ladder, 2x 3T3, 2x MCF7, 2x 14C and 2x A431.

No activation of HER3 by anti-HER3 VHHs

For the activation and completion study the VHHs E11, O6C6 and O7C4 were used. The non treated lanes and lanes with neuregulin alone are used as control lanes, to check the standard amount of HER3/actin or pHER3/pAkt. In figure 5 the results for the MCF7 cell line are showed. From the left membrane can be concluded that the different VHH do not activate HER3. This because only in the lanes where neuregulin was added there is a band for pHER3 (about 185 kDa) and pAkt (about 60 kDa; red) visible. The right membrane shows that the amount of cells per lane is equal (red = actin) and that the MCF7 cell line indeed has got a lot of HER3. It also shows that the amount of activation is lower in presence of E11 in comparison with the lanes with only neuregulin, indicating that there is some competition between E11 and neuregulin.

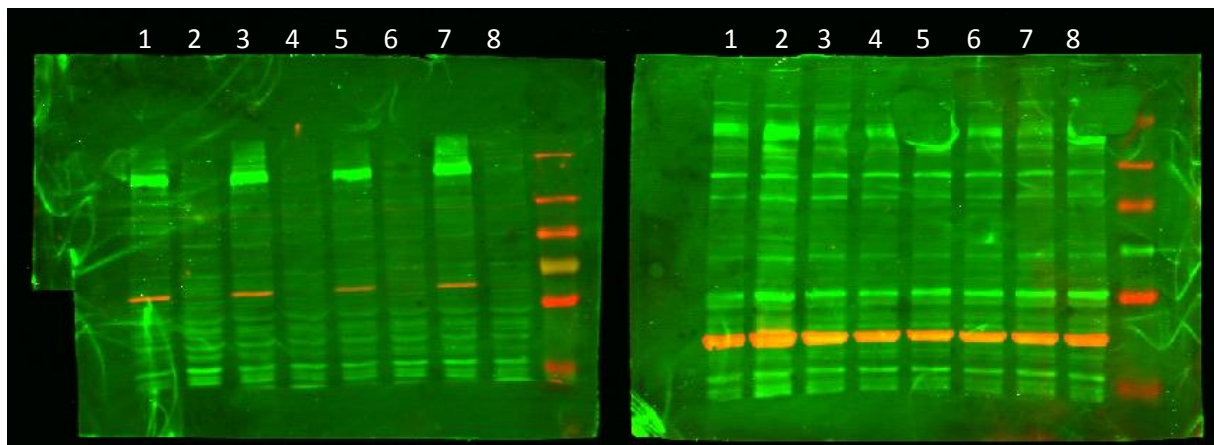


Figure 5: Odyssey scan of membrane obtained from Western blot. The left membrane is incubated with pHER3 and pAkt anti bodies and the right is with anti HER3 and actin antibodies. The content of the lanes from left to right is O7C4&NRG, O7C4,O6C6&NRG,O6C6,E11&NRG,E11,NRG and one non treated lane.

Legend of figure 5:

1: O7C4 and NRG	5: E11 and NRG
2: O7C4	6: E11
3: O6C6 and NRG	7: NRG
4: O6C6	8: non treated

Binding assay 1: E11 binds the best to HER3, without detectable competition with neuregulin

In figure 3 are the results from the Odyssey scan of the 96 well plates used in the first binding study. The content of the wells used in the black rectangle:

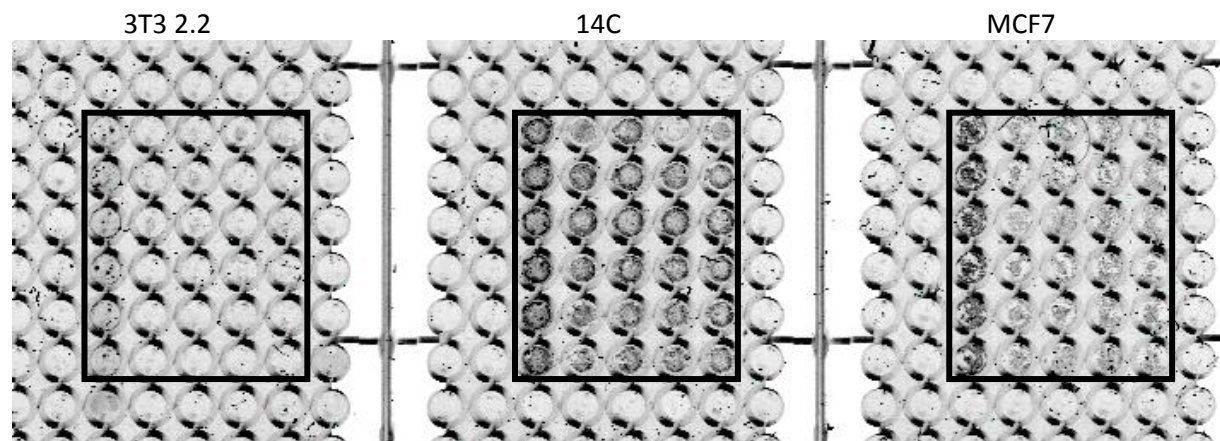
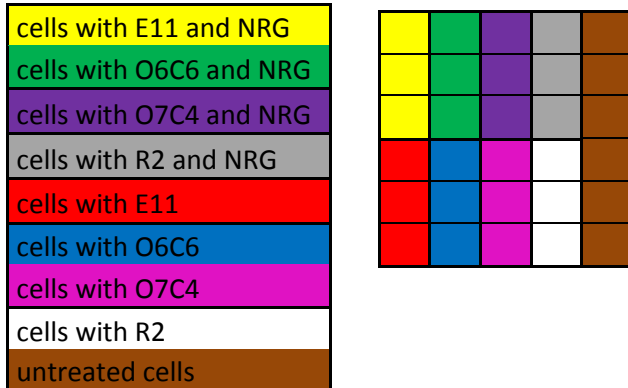
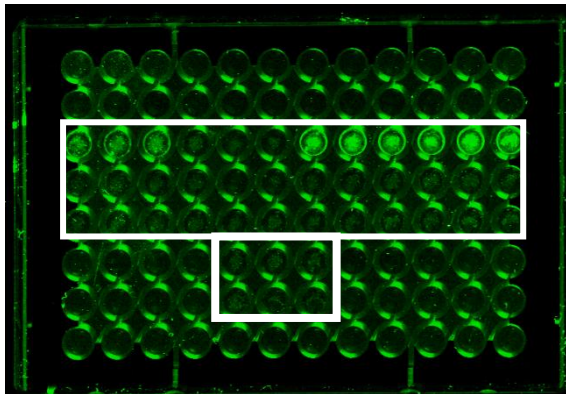


Figure 3: Odyssey scan with grayscale. From left to right is in the well plates; 3T3 2.2, MCF7 and 14C.

The values of intensity per well were measured with the Odyssey scanning program and transferred in to three separate graphs. The tables with the values (2,3,4) and graphs (1,2,3) for the different cell lines suggest that E11 is binding the best to HER3. The presence of neuregulin does not seem to affect the amount of VHH bound. This also suggests that the VHH do not compete with neuregulin, which is also clearly visible with E11 (although preliminary results suggested otherwise). R2 is a VHH that is known for not binding to HER3 and it will be used as a negative control, therefore the intensity of these wells subtracted by the intensity of the “background wells” (the untreated wells with only the primary and secondary antibody) is a-specific binding.

Binding assay 2: E11 shows an increasing binding with higher concentrations

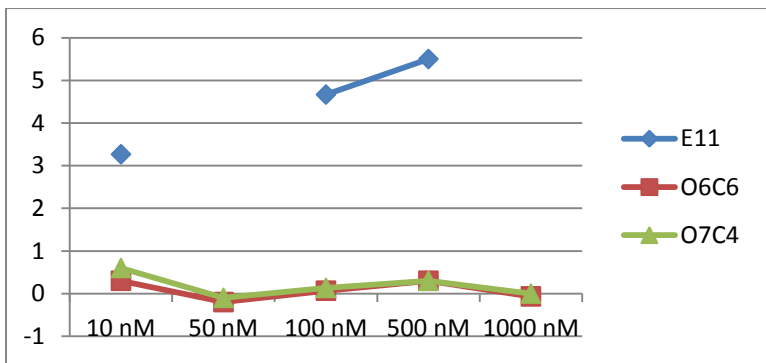
For the binding study three VHHs were used (E11, O6C6 and O7C4). In figure 8 a Odyssey scan is shown together with an overview of the content of the wells. The values of the image were then future analyzed in graph 5.



10 nM	50 nM	100 nM	500 nM
E11	NT	E11	E11
O6C6	O6C6	O6C6	O6C6
O7C4	O7C4	O7C4	O7C4
	O7C4		
	O6C6		
	1000 nM		

Figure 8: Odyssey scan of a 96-well plate containing cells with dilution of E11, O6C6 and O7C4

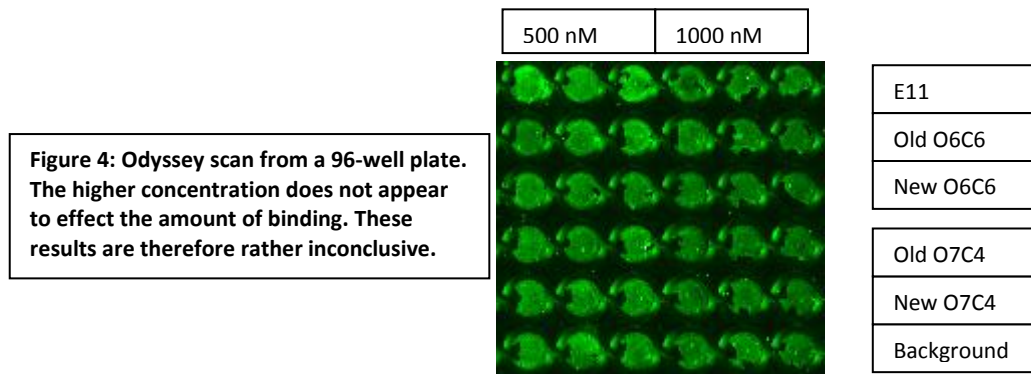
In graph 5 the data obtained from the plate is shown. The VHHs O6C6 and O7C4 do not show any significant binding in this study. E11 on the other hand does show an increasing binding when the concentration is higher. This means that the affinity for HER3 is much higher with E11 than the other VHHs.



Graph 5: A graph of the intensity of the dilutions subtracted by the background. There were only three measuring points with E11 and five with O6C6 and O7C4.

Binding assay 3: little difference in the binding of HER3 is found between the old and new VHHs

One of causes of the little binding of O6C6 and O7C4 could be the age of the VHHs. To exclude this, a new assay was made with the freshly made VHHs. The results are shown in figure 4, it can be seen that the higher concentration does not result in a higher intensity of signal. This would mean that the concentration does not affect the amount of binding of the VHHs, this is not in concordance with the other results. There also appears to be hardly any difference between the old and the new VHHs.

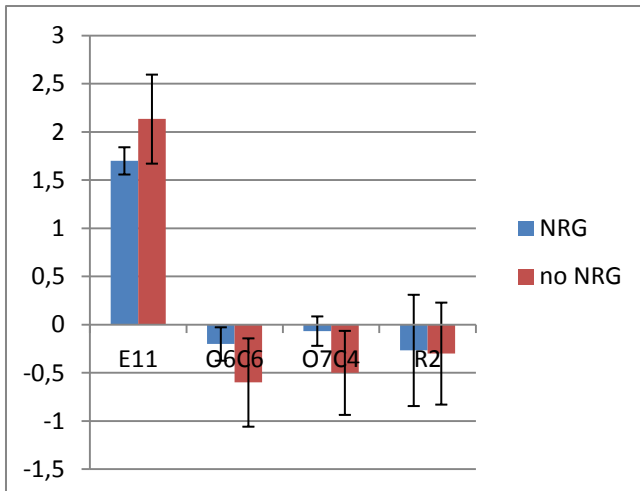


Binding assay 4: new VHHs bind better to the ECD of HER3 than old VHHs

The study with the extracellular domain was done because of the little binding found with the cells of different cell lines in the other binding studies, although in previous studies (by Smiriti Raghoenath) the outcome was positive with the extracellular domain. To exclude problems with the VHHs, this study was repeated. The results from the binding study with the extracellular domain are shown in table 1. The values of the lower concentration are in almost all of the cases higher. This is not what would be expected and there was no clear explanation found. However a great difference was observed between the intensities of old and new batches of O6C6. The results for the new O7C4 are not very clear either.

	E11	O6C6 (old)	O6C6 (new)	O7C4 (old)	O7C4 (new)
1 μ M	0,012	-0,016	0,109	-0,016	-0,028
500 nM	0,021	-0,005	0,222	0	background

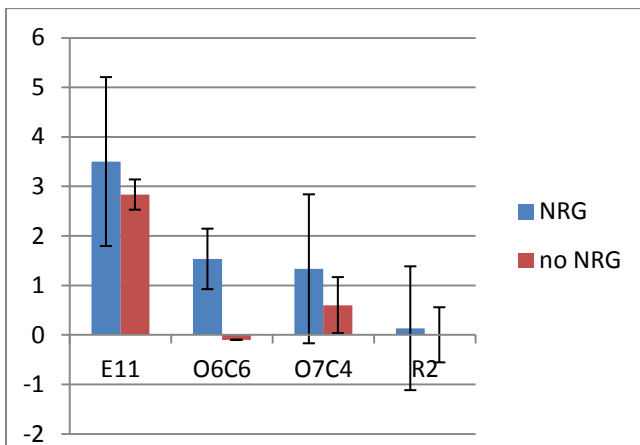
Table 1: Intensity values from ELISA of well plate with the extracellular domain from HER3. The results suggest that a lower concentration give a higher intensity, which is not what is expected. There is a clear difference between old and new O6C6.



Graph 1: intensity from Odyssey scan on HER3 binding of 3T3 2.2, corrected for background and error.

3T3 2.2	E11	O6C6	O7C4	R2	background
NRG	7	5,1	5,3	5,6	5,2
NRG	15,7	5,2	5,5	5	5,6
NRG	7	5,3	5,2	4,8	5,4
no NRG	8,2	4,8	5,1	5,3	5,8
no NRG	7,3	4,8	4,9	5,2	4,9
no NRG	7,2	4,9	4,8	4,9	5,6

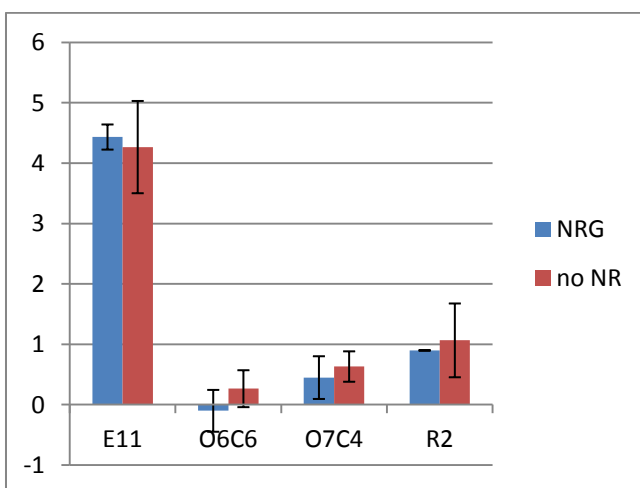
Table 2: The data per well, red data is excluded.



Graph 2: intensity from Odyssey scan on HER3 binding of 14C, corrected for background and error.

14C	E11	O6C6	O7C4	R2	background
NRG	12,5	8,5	10	5,8	7,1
NRG	12,1	11,3	10,3	9,8	9,1
NRG	11,7	10,6	9,5	10,6	9,6
no NRG	13,1	10,5	10,8	10,1	10,6
no NRG	12,5	9,5	17	9,5	9,6
no NRG	12,2	9	10,1	9,7	9,1

Table 3: The data per well, red data is excluded.



Graph 3: intensity from Odyssey scan on HER3 binding of MCF7, corrected for background and error.

MCF7	E11	O6C6	O7C4	R2	background
NRG	11	6,8	22,7	7,4	6,5
NRG	11,1	6,2	6,7	15,2	6,5
NRG	10,6	6,1	7,1	7,3	6,4
no NRG	11	6,5	6,3	7,1	5,9
no NRG	10,6	6,7	7,4	6,9	6,5
no NRG	10,5	6,9	7,5	8,5	6,9

Table 4: The data per well, red data is excluded.

Conjugation of IRDye800CW to E11 was successful.

The study on the behavior of VHHs conjugated with a fluorophore group (green) was done after a quality control of the binding between the fluorophore group and the VHH. In figure 6 data is shown from a gel with from left to right: ladder, R2, conjugated R2, 7D12, conjugated 7D12, E11, conjugated E11, Cetuximab, conjugated Cetuximab, EGA1. The data for EGA1 is left out for this study. The data in figure 6 shows that the fluorophore group is bound to the VHH. In the white rectangle are the lanes with E11 (left) and conjugated E11 (right), these are the lanes import for the following binding assay (5). The lane of conjugated E11 is not visible, this made be a result of to little protein in the sample.

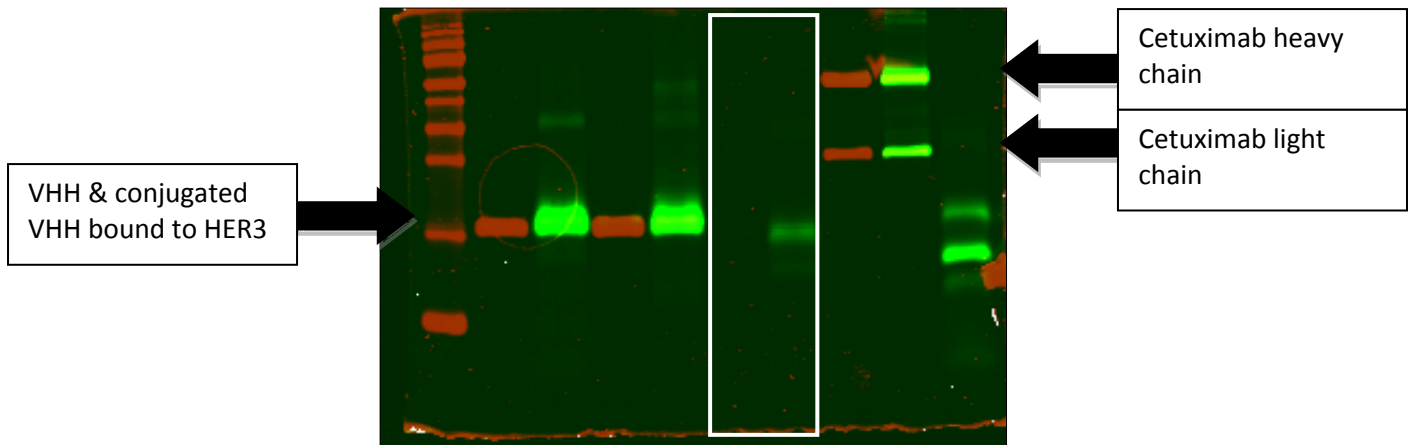


Figure 6: A odyssey scan of a western blot with from left to right; ladder, R2, conjugated R2, 7D12, conjugated 7D12, E11, conjugated E11, Cetuximab, conjugated Cetuximab, EGA1.

Binding assay 5: the conjugation of the VHH with a fluorophore group does not affect the binding to HER3 expressing cells

In this binding study a dilution series with conjugated E11 was made. In figure 7 is the image made with Odyssey of the plate shown. The content of the wells in the white rectangle is explained with the colored boxes. The data shows a decreasing intensity when the concentration decreases.

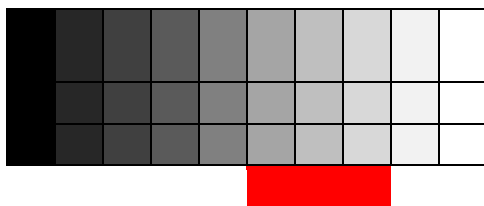
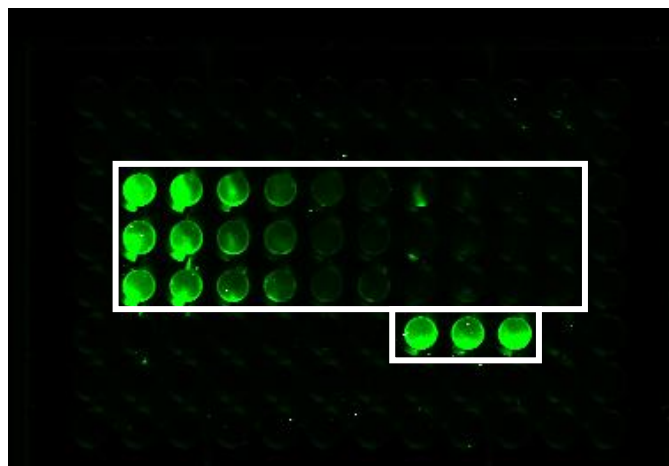
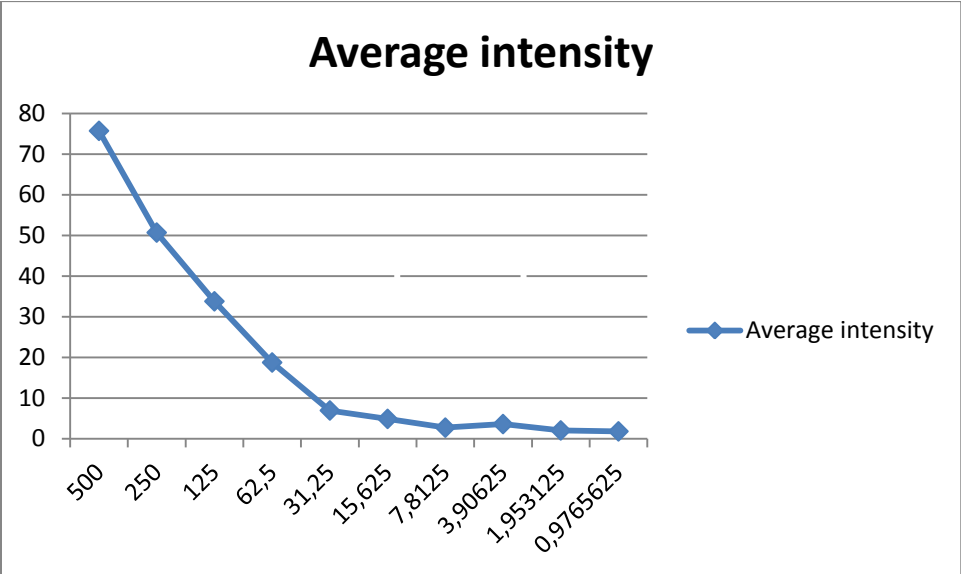


Figure 7: Odyssey scan of a 96-well plate containing triplets of 10 dilution (500 nM to 0.98 nM in 9 equal steps) of conjugated E11.

500 nM
250 nM
125 nM
62,5 nM
31,25 nM
15,63 nM
7,81 nM
3,91 nM
1,95 nM
0,98 nM
non treated



In graph 4 the data of figure 7 is shown. It is clear that the intensity is dependent of the degree of dilution. A plateau is not reached with the highest concentration. The affinity of the conjugated E11 is not very high and a higher concentration is needed to determine the “peak-affinity”.



Graph 4: A graph of the intensity of the dilution series of conjugated E11 subtracted by the background. The affinity is higher with a higher concentration.

6.4: Conclusion

The results point out that not all the VHHs bind as good with the HER3 receptor as they did in previous studies with the extra cellular domain of HER3. E11 is the best binder, but from our results we cannot conclude that the affinity is high enough for the VHH to be used as imaging tool for breast cancer. The results for the difference are not clear, one of the possibilities was that the VHHs lost their affinity after a certain amount of time. This was not excluded with the study with “old” and “new” VHH, so this study can be done again in the near future. A positive outcome is that the VHHs do not activate HER3, which would not be suitable for either imaging or therapeutic purposes.

For future research it would be interesting to find out how high the saturation plateau for E11 and other VHHs is. It would be interesting to check more VHHs and more cell lines, in order to find the VHHs with the highest affinities. What cannot be proven in our research is that the VHHs bind only to HER3. It would also be an option to do more research on the specificity for HER3 (of E11).

Chapter 7: possible follow-up studies

In this practical study we have not been able to prove that the VHHs that were used are binding specifically to HER3. In future studies this proof could support the possibility for a VHH targeted for HER3. Only four different VHHs (apart from controls) were used with the different studies performed in this thesis. Because of the non-ideal outcome, regarding the affinity, looking at more and different VHHs in future studies might provide better results. As well as with the VHHs, only a few cell lines were selected, for future studies it might be useful to perform studies with more cell lines. Using cell lines with different amounts of expression of HER3 might provide an insight on the effect of the amount of HER3 on the affinity of the different VHHs.

Acknowledgements

I would like to thank the department of cell biology for the opportunity to do my practical work for my thesis at their facilities. I would like to thank in particular Sabrina Oliveira for guiding me through the literature and practical research I have done.

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