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Characterization of antigen binding potential of a novel antibody-HRP conjugate designed for antibody-directed enzyme prodrug treatment of breast cancer cells

Bachelor's Thesis (12 ECTS)

Curriculum Science and Technology

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Characterization of antigen binding potential of a novel antibody-HRP conjugate designed for antibody-directed enzyme prodrug treatment of breast cancer cells

Abstract:

The aim of this paper is to study the newly designed antibody-conjugate, Trastuzumab-HRP, for binding ability to ErbB2 protein on human breast and lung cancer cell lines using western blotting, immunoprecipitation and immunofluorescence methods. The first part of this bachelor thesis is theoretical and is processed on the basis of the background research. Breast cancer problem, risk factors, general treatment, ADEPT therapy, ErbB2 protein as tumor target antigen, trastuzumab antibody description are included in the first part. The second part of the thesis is specialized and is processed on the basis of the experiments with Trastuzumab-HRP involving breast and non-small cell lung cancer cell lines: AU565, MCF-7, A549, H1299 and H23. This part consists of results of western blotting method, in which trastuzumab was unable to sufficiently detect the ErbB2 protein and the results of alternative method, immunoprecipitation, which showed the capability of trastuzumab to bind to its specific antigen. The immunofluorescence method demonstrated the ability of the novel antibody to recognize and bind to the native form of protein.

Keywords:

Breast cancer, antibody, antibody-conjugate, ErbB2, trastuzumab, HRP

CERCS: T490-Biothechnology

Rinnavähi rakuliinide antikeha-suunatud ensüümi prodrug teraapiaks disainitud uudse antikeha-HRP konjugaadi antigeeniga seondumise omaduste kirjeldus

Lühikokkuvõte:

Selle töö eesmärgiks oli testida uudset disainitud antikehakonjugaati, Trastuzumab-HRP, ja selle võimekust seonduma ErbB2 valguga inimese rinnavähi ja kopsuvähi rakuliinides, kasutades selleks western bloti, immuunopretsipitatsiooni ja immufluorestsentsi meetodeid. Selle bakalaureusetöö esimene osa on teoreetiline ja kujutab endast ette taustauuringu tööga seotud teemadest – rinnavähk, selle riskifaktorid ja olemasolevad ravivõimalused, ADEPT teraapia põhimõtted, ErbB2 valk kasvaja antigeenina ja trastuzumab antikeha kirjeldus on käsitletud esimeses osas. Teine osa on pühendatud praktilisele tööle trastuzumab-HRP antikehaga ja inimese rinnavähi rakuliinidega - AU565 ja MCF-7 ja inimese kopsuvähi rakuliinidega - A-549, H1299 ja H23. Selles osas on kirjeldatud tulemused western bloti analüüsist, milles trastuzumab ei olnud võimeline detekteerima oma antigeeni – ErbB2 valku, ja tulemused alternatiivsest meetodist immuunopretsipitatsioonist, mis tõestasid, et trastuzumab-HRP on ikkagi suudab ikkagi detekteerida ja seonduda oma märklauale. Immunofluorestsents meetodi abil sai näidatud, et uus antikeha on võimeline seonduma ka ErbB2 valgu natiivse vormiga elusrakkudel.

Võtmesõnad:

Rinnavähk, antikeha, antikehakonjugaat, ErbB2, trastuzumab, HRP

CERCS: T490-Biotehnoloogia

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List of abbreviations

ADEPT- antibody-directed enzyme prodrug therapy

BMI – body mass index

BRAF - B-raf gene

CPG2 – carboxypeptidase g2 (guanosine)

ECD – extracellular domain

EGFR - epidermal growth factor receptors

ER- estrogen receptor

ErbB2 - erythroblastosis oncogene B

HER2 - human epidermal growth factor receptor

HRP - horseradish peroxidase

IAA - indole-3-acetic acid

mAb – monoclonal antibody

PR- progesterone receptor

WHO - The World Health Organization

INTRODUCTION

Cancer has received much attention in the last decades due to significant emergence rates all over the world. The mortality level by breast cancer reached approximately 1 million worldwide in 2018 according to the American Cancer Society data. Therefore, the clinical and experimental researches have been extensively pushing forward to create new treatments and prevention approaches. Among them are anti-HER2 therapies, anti-EGFR tyrosine kinase inhibitors, anti-BRAF agents. Nevertheless, there still remains a need for a novel treatment method, that has minimal toxic side-effects and simultaneously, proposes effective elimination of disease.

In this paper we examine the newly designed human monoclonal antibody trastuzumab-HRP, which is aimed at binding to ErbB2 protein, which is quite often overexpressed in breast cancer cells. The development of chemotherapy drug conjugate allows to raise the selectivity to tumor cells. The principle of action is based on ADEPT therapy *with IAA/HRP* that activates cytotoxicity against breast cancer cells without damaging normal cells.

1.1. Breast cancer

1.1.1 Occurrence rate and statistics

The changes in a single cell that are followed by uncontrolled growth and spread of abnormal cells is called cancer. The transformation from a normal cell into tumor cells is studied by branch of medicine called oncology. (Rivenbark A.G. et al., 2013)

Most of the cancer deaths are caused by chronic myeloid leukemia, lung, breast, liver, colon cancer and melanoma. Numerous global researches showed that more than 40% of cancer deaths could be prevented. Still, according the WHO data and statistics, the representation of cancer on global scale was accounted for 9.6 million deaths in 2018, comparing to 8.2 million human deaths in 2012. (https://www.europadonna.org)

Besides the skin cancer, breast cancer is the most common malignancy diagnosed among female population of Europe, America and some Asian countries. In 2018, the estimated deaths from breast cancer were 627,000 (15% of all cancer female deaths) and among which European region women were estimated 562,500. (https://www.europadonna.org)

During last 20 years there was noticed a strong upward trend in the number of cancer patients due to increases in life expectancy and way of life, especially those who live in big cities or megapolises.

1.1.2 Causes and risk factors of breast cancer

The lifestyle choices constitute of risk factors for breast cancer diagnosis, as they are associated with general neglecting of health. Among modifiable risk factors, is excessive alcohol consumption (from 3-6 drinks per week), which is directly linked with increasing level of breast cancer chances. Another risk factor highly associated with obesity and its consequent diseases that is specifically critical for postmenopausal women (physical activity for maintaining the same level of BMI (body mass index) through life decreases threat by 5 %). Another risk for disease initiation is radiation caused by medical treatment and nuclear explosion. Generally, after the treating for Hodgkin’s disease and receiving therapeutic radiation, the chances for the patient to develop breast cancer are greater. (Shah R. et al, 2014)

Along with other groups of risk, endogenous hormone exposure and subsequent reproductive alterations have a significant role in development of cancer. This has been accompanied by early menarche, delayed age at first full term pregnancy, delayed age at menopause, high testosterone level, failure to breastfeed. In addition, the genetic predisposition, personal and family history, breast pathology and others have been associated with an increased risk of receiving this diagnose. (Shah R. et al, 2014)

1.1.3 Standard treatment and barriers

It is still very challenging to treat and cure breast cancer. First of all, it is not a single disease, there are hundreds of different types of cancer, moreover, there are more than 400 different gene mutations implicated in the development of the disease, and finally, it displays major inter- and intratumor heterogeneity. (Duffy M., 2013)

Heterogeneity of this disease is determined by plenty of morphological, clinical and molecular features. These criteria are used to classify breast cancer according to grade - low to high grade defined from lump growth, change in breast shape, presence of dimples and fluid coming from nipple, tumor size, TNM (tumor, node, metastasis) staging system, immunohistochemical markers expression (ER, PR, HER2). (Rivenbark A.G. et al, 2013)

The transition from guaranteed death sentences to manageable chronic diseases became possible only with the overcoming of the above-standing complexities by qualitative researches, such as chemical molecular diagnostics and biomarker discoveries. (Roskoski R.Jr., 2014)

An increasing number of studies have found that breast cancer types are grouped into five categories: (1) luminal A (ER^+), (2) luminal B (ER^+/HER2-enriched), (3) $HER2^+$ (HER2-enriched), (4) basal-like, and (5) normal-like. The most common subtypes are Luminal A with a frequency around 30% and Luminal B that represented in approximately 20% of patients. Both of them (ER^+ subtypes) show considerably long-term survival capacity – 80% - 85% 5-year survival in comparison with $HER2^+$ and basal-like (ER^- subtypes) which estimated at 50-60% respectively. Both triple-negative and basal like types of cancer considered to be aggressive tumors, so they spread very quickly and have poor prognosis. (Rivenbark A.G, 2013)

Consequently, the ability to survive either of those breast cancer subtypes is closely linked to the effectiveness of treatment options that can be divided to local and systematic. The local treatment aims to remove breast cancer without affecting the whole body by surgery or

radiation. The systematic treatment comprises of chemotherapy, hormone therapy, targeted therapy and immunotherapy. Clearly, some kind of treatments target better specific types of cancer and therefore there is no universal method for approaching breast cancer. For instance, the therapy in the form of anti-estrogen hormonal treatment and sequentially with adjuvant chemotherapy plays an important role in elimination of ER+ subtypes of breast cancer. By contrast, the medication of $HER2^+$ (ER^-) does not include hormonal therapy due to its low effectiveness on this subtype, so it is based on assembly of targeted drugs and cytotoxic chemotherapy. (Rivenbark A.G, 2013) (https://www.cancer.org)

One of the most frequent obstacles for effective treatment is development of resistance, which is the main cause of death in most patients with cancer. The mechanism of secondary mutations in target protein, which interfere with drug binding, is an example of this kind of obstacles. A different development of resistance to molecularly targeted therapies is the emergence of alternative signaling pathways, which bypass the blocked target. Thus, the avoiding of resistance mechanism often require combined treatment with different therapies. (Duffy M, 2013)

Another difficulty that is likely to occur is the cost of treatment. Total spending on cancer care in USA is estimated to reach US$157 billion by 2020. Even though the molecularly targeted therapies are currently revolutionizing the treatment of certain cancers, their costs are very high. Also, the drug development itself costs approximately one billion US dollars and can take around 10 years to complete. Finally, the problem is aggravated by limited effectiveness of some treatments. The small gain in survival is associated with an increased risk of toxicities such as interstitial lung disease, diarrhea, and therapy-related death. (Duffy M, 2013)

1.1.4 New treatment approaches. Biomarkers.

Biomarkers in oncology are divided into diagnostic, prognostic, treatment and prevention subgroups based on the purpose of the cancer signature characterization. Examples of these molecularly targeted biomarker therapies are HER2/neu blockage in HER2/neu-positive breast cancer and epidermal growth factor receptors (EGFR) inhibition in EGFR-mutated lung cancer. (Kalia M., 2015)

Among the most successful strategies in targeting driver genes involved in cancer developments are imatinib (for the treatment of chronic myeloid leukemia), anti-HER2 therapies (trastuzumab, pertuzumab, lapatinib) to treat breast cancer, anti-EGFR tyrosine kinase

inhibitors (gefitinib, erlotinib) to treat non-small cell lung cancer and anti-BRAF agents (vemurafenib and dabrafenib) to treat melanoma. (Duffy M., 2013)

HER2 gene expression in breast cancer is both predictive and prognostic biomarker. HER2-negative tumors do not respond to novel trastuzumab treatment, but the antibody has specific therapeutic effect in HER2-positive tumors. Thus, HER2/neu acts as a predictive biomarker of a potential trastuzumab response in a patient with newly diagnosed breast cancer. It can also be called a prognostic marker as HER2 positive patients carry a worse survival outcome than patients without HER2/neu. (Kalia M., 2015)

1.2. ADEPT therapy

Prodrug-generating technology is aiming to overcome some of the limitations of early antibody-drug conjugates and lack of tumor selectivity, and it has greatly progressed as effective cancer therapy since the end of the last decades. The primary prerequisite to the new concept was attempted to search for utilization enzymes within tumors that convert low toxicity prodrug to a cytotoxic agent, but no unique tumor specific enzymes were found. So, the ADEPT (antibody-directed enzyme prodrug therapy) concept has been designed in 1987, to solve this problem by delivering of the unique enzymes to tumor site. The concept relies on antibody-enzyme conjugates directed at tumor-associated antigens to achieve site-specific activation of prodrugs (Melton R, 1996)

There is a variety of enzymes that can be used in ADEPT, including CPG2, CPA, alkaline phosphatase, glycosidase, penicillin amidase, cytosine deaminase. Selected enzyme and a suitable prodrug pair can be used with any solid tumor type by attaching enzyme to a relevant targeting antibody, or the same way a specific targeting antibody can be used with different enzyme and prodrug pairs. So far, only the researches with CPG2 enzyme were carried out in clinical application by conjugating it to a non-internalizing antibody directed at secreted tumor associated carcinoembryonic antigens by using humanized or fully human antibodies (α-CEA). (Sharma S. et al, 2017), (Napier M.P. et al, 2015), (Melton R, 1996)

As any other therapy, ADEPT concept's results are analyzed for toxicity, efficacy and immunogenicity. The major limitation of CPG2 system is the immunogenic potential of enzyme, because generation of neutralizing antibodies will limit the use of repeat cycles of ADEPT in patients. As cyclosporine induces toxicity, other approaches to make enzymes non-

immunogenic were designed. These included directed evolution of enzymes, application of algorithms to optimize for enzyme function and low immunogenicity, or selection from combinatorial libraries (source of components offers the possibility of producing virtually non-immunogenic constructs). (Sharma S. et al, 2017), (Napier M.P. et al, 2000)

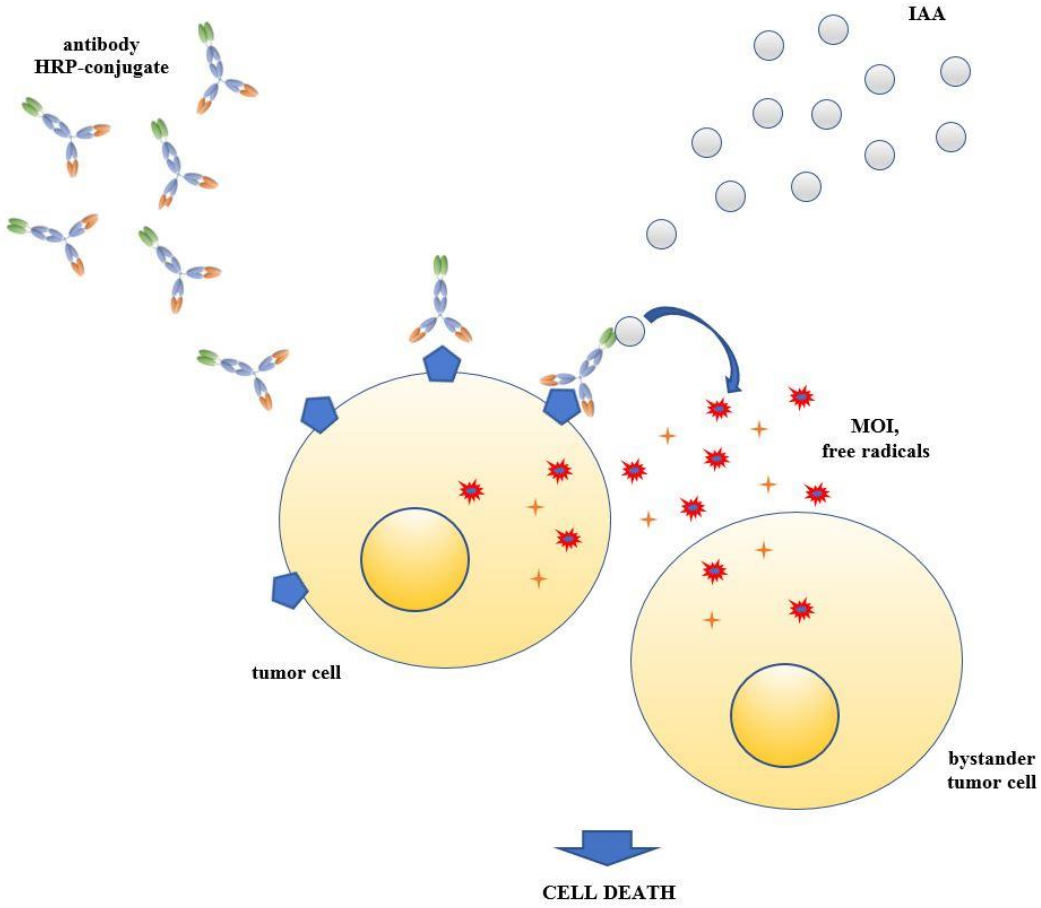

Fig.1 Principle of HRP/IAA ADEPT therapy. *The process begins with the administration of antibody HRP-conjugate that binds and accumulates at the surface of specific tumor-associated antigen-expressing cells. Then, a nontoxic prodrug IAA is added and when it reaches the tumor cells, with bound antibody HRP-conjugate, it is oxidized and as a result, MOI and free radicals are released. Also, since the size of free radicals and MOI is very small, they are able to penetrate deeper into the tumor tissue and kill the bystander tumor cells* (K. Gildemann *et al*, not yet published)

One more possible prodrug/enzyme combination for targeted cancer therapy is indole-3-acetic acid (IAA) as a prodrug and horseradish peroxidase (HRP) as an enzyme. IAA is a plant growth hormone, from the auxin family. It is involved in the regulation of cell differentiation and cell growth. Despite the hormonal role of IAA in development of plant, it has radical fragmentation

chemistry, which results in formation of fragments with ring-substituted phenylhydrazine and ketone and the decarboxylated radical cations (indolyl, skatolyl, peroxyl) after being oxidized by HRP. (Kim D.S., 2006) The essential aspect in IAA/HRP system is monoamine oxidation induced by HRP. IAA is promising as a prodrug, because of reactivity of carbon-centered free radicals towards DNA and subsequent products - methyleneoxindole (MOI) towards nucleophiles, such as thiols and histones. Localization of MOI nearby the cancer cells generates the adducts and strand breaks in DNA, and it leads to cell death (Wardman P., 2002)

The unique role in ADEPT therapy plays bystander effect. It provides the potential to obtain efficient therapeutic effect of the tumour cells without target antigen expression from already eliminated directly-targeted tumor cells. (Bremer E. *et al*, 2004)

The undeniable advantages of this kind of therapy is very low toxicity of prodrug in humans and reasonable pharmacological properties. Although, prodrug or enzyme treatment alone have no significant effect under the used conditions, its combination shows high cytotoxicity. (Kim D.S., 2006) The properties that serve as important criteria for using HRP enzyme in ADEPT are following: no equivalent activity in humans (human peroxidases are much less active in oxidizing IAA derivatives than HRP), high specificity for the prodrug (IAA and derivatives are much better substrates for HRP than related biogenic indoles), it has no substrate requirements, it is active at neutral pH (cytotoxicity happens at physiological pH), the reaction is irreversible, thermal and conjugation stability, affordable price. (Napier M.P., 2000), (Melton R, 1996)

According to conducted trials in colorectal cancer treatment, the ADEPT therapy has low short- and long-term toxicity on normal tissue and has shown antitumor activity on breast carcinoma. (Melton R, 1996) The clinical trials with ADEPT for general feasibility showed that it could interact in-vivo to give therapeutic benefit indicated by encouraging response in patients with advanced metastatic colorectal cancer, who failed on all other treatments and were only expected to survive for less than 8 weeks. Overall, the observed studies demonstrated that using ADEPT as multifunctional recombinant fusion protein could be administrated as repeated therapy with acceptable toxicity. (Sharma S. et al, 2017)

1.3 HER2

1.3.1 General description of ErbB2

ErbB2 (HER2, HER2/neu, CD340, NEU, EGFR2) is one of the most important biomarker for cancer of ovary, endometrium, breast, fallopian tube, cervix in overexpressed form (resulted from gene amplification). This oncogene is located in human chromosome 17q21 and encodes a 185-kDa transmembrane tyrosine kinase glycoprotein that resembles a receptor for epidermal growth factor. (Cirisano F. et al., 1996)

The human EGF receptor family consists of four members that belong to the ErbB lineage of proteins (ErbB1-4, or HER1-4). The idea of pathogenical role of HER2 receptor in cancer was carried out by Schechter et al. in 1984, who found that Neu oncogene is related to the rat HER2 gene of the EGF receptor family. The extracellular domain of HER2 is divided into 4 parts (domains I and III participate in ligand binding, domains II and IV participate in disulfide bond formation). (Roskoski R.Jr., 2014)
The activation of this protein induces proliferation and intracellular signaling pathway, including the MAPK and PI3K-Akt pathways. The MAPK activation of transcription factor includes transphosphorylation of cytosolic tyrosine residues after the formation of heterodimers. Due to fact that ErbB2 has no need in any specific ligand, there are various combinations of ErbB2 dimerization as homodimers and heterodimers. (Marques M. et al, 1999) (Lukas C. Amler et al, 2012)

The findings of the role of ErbB receptors show that they are functioning mostly from plasma membrane, but these receptors are also translocated to the nucleus where they participate in cell signaling pathways (angiogenesis, cell adhesion, cell motility, development, organogenesis) that consist of several interconnected and overlapping modules. (Roskoski R.Jr., 2014)

Due to the fact the overexpression of ErbB2 is controlled by rate of gene transcription, it is important to stress that the ErbB2 expression is regulated by AP-2 transcription factor in human breast cancer epithelium as cell growth and differentiation controller. Also, the transcriptional extension can be provided by active participation of steroid hormones. They function as intermediaries to position hormone-receptor complexes to DNA specific regulatory sites and maintain mRNA transcripts. (Cirisano F. et al., 1996)

1.3.2 ErbB2 as tumor target antigen

The investigation of epidermal growth factor mutations or its increased expression played crucial role on the assumptions made in breast cancer research. ErbB2 is one of the most studied receptor protein-tyrosine kinases, because it regulates nearly every aspect of cell biology, like transcription, apoptosis, cell cycle progression, cytoskeletal rearrangement, differentiation, development, immune response, nervous system function. Dysregulation of protein kinases carries the risk of cancer and other disorders. (Roskoski R.Jr., 2014)

The expression of ErbB2 in normal tissue remains at low level in certain epithelial cell types. The quantification method demonstrated that ErbB2 overexpression or amplification is correlated with metastatic breast cancer in 20-30% of the cases. The triple-negative breast cancer lacks a therapeutic target comparing to basal-like and it explains why identification of ErbB2 as cancer target is not always used. It was identified that the expression of this oncogene does not relate to histological or nuclear grade, estrogen or progesterone receptor status. (K. McKeag and C. Perry, 2002), (Cirisano F. et al., 1996)

Nowadays, there are three ways of targeting of cancer in patients with overexpressed ErbB2 tumors: application of HER family antibody conjugates, bispecific antibodies, and novel targeted combinations. For these purposes, the biomarker has high potential for extending the quantity of robust therapeutic methods. The treatment introduces the application of HER family monoclonal antibody conjugates, bispecific antibodies and novel targeted combinations, antisense DNA, adenovirus-mediated E1A delivery. (Lukas C. Amler et al, 2012) The antibody treatment has effect through the activation of immune and inflammatory system, or it is in charge of blocking physiological function of the ErbB2 receptor.

In 15%–25% of invasive breast cancer the clinical trials showed a favorable response of trastuzumab to inhibit the overexpression of ErbB2. The progression-free survival and disease control could be enhanced by blocking of both the intra- and extracellular domains of ErbB2 receptor by lapatinib and trastuzumab (respectively). (Kalia M., 2015)

1.4 Trastuzumab

1.4.1 Design and development

In order to identify new effective strategies to treat breast cancer, we need to target the juxtamembrane portion of the ECD of the human EGFR2 protein, ErbB2 receptor, which is overexpressed in the proliferating tumor cells, including 25-30% of the metastatic breast cancer. (Michael C. Perry, 2008:56), (Dr. Hudis, 2007) Thus, the inhibition principle of tumor growth by antibodies was invented. (Baselga, 2001)

Researches with the anti-p185HER2 murine monoclonal antibody 4D5 that immunize mice cells had to be changed due to immunogenic potential that limits usage of MAbs in humans. In order to improve clinical efficacy of the murine Mab 4D5, all the mouse-derived components of IgG molecules were replaced, except the antigen-binding region, with human counterparts and the result product was called trastuzumab. (K. McKeag and C. Perry, 2002)

Today we consider humanized mAb – trastuzumab as:

1) first-line therapy *in combination* with chemotherapy medication increases time of disease progression (at a median time of 7.4 months versus 4.6 months; or 25.1 vs 20.3 months). It has 1-year survival compared with chemotherapy alone when co-administered to patients with metastatic HER2-positive breast cancer. (K. McKeag and C. Perry, 2002), (M. Goldenberg, 1999)
2) *single agent* in second- or third-line treatment that provides durable objective tumor responses (15% of patients) and notably, 21% of patients were free of disease progression at 6 months. (J. Baselga, 2001)

Trastuzumab was approved in 1998 as US FDA for the treatment of metastatic breast cancer overexpressing ErbB2. (Baselga, 2001)

1.4.2 Mechanism of action

The principle mechanisms that determine trastuzumab are: blocking of dimerization of ErbB2-receptor, processing to degrade the receptor, inhibition of shedding of the ErbB2 ectodomain which serves as monitoring marker, and the immune activation of antibody-dependent cell-mediated cytotoxicity that leads to tumor-cell death. (Dr. Hudis, 2007)

The HERA trial has shown the benefit of trastuzumab, after completion of the primary therapy (surgery, chemotherapy), by testing 2 groups of women (around 1700 for each) with HER2-positive breast cancer. Trastuzumab reduced the rate of recurrence by approximately 50 %. The undeniable advantage in using trastuzumab lies also on the fact that it is effective regardless of the type of chemotherapeutic regimens received before treatment. The analysis showed that administrating one-year locoregional therapy and combination with other therapy for women that fit criteria used in the HERA trial are suggested as standard care. (M. Piccart-Gebhart et al, 2005), (J. Specht et N. Davidson, 2017)

In broad biological terms, toxicity as intolerant reaction refers to significant damage that arises in organism and accompanied by health dysfunction. According to statistics, 84% of patients experiences at least one adverse event related to trastuzumab therapy (14 % among them were considered severe). (K. McKeag and C. Perry, 2002) Researches who have looked on safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer, have found some treatment-related adverse events like chills, asthenia, fever, pain, and nausea, vomiting, increased cough, diarrhea, headache, dyspnea. (C. Vogel et al, 2002), (M. Goldenberg, 1999)

2. THE AIMS OF THE THESIS

The breast cancer therapy has obtained significant extent across the European Union, but it still fails to guarantee complete recovery of patients, even with all the different molecularly targeted therapies currently available. Moreover, huge amounts of money are spent every year on treating patients with these therapies. Nevertheless, the effectiveness and sensitivity of these methods and, in some cases, toxicity vary from patient to patient. In order to achieve greater scale of treatment efficiency and cost-effectiveness, the search for new therapies that are able to postpone, or cure cancer is never stopping.

Trastuzumab antibody genetically conjugated with HRP enzyme, for studying of ADEPT approach in our lab *in vitro* on breast and lung cancer cell culture, was designed and produced in Icosagen AS for our research. It is important to emphasize that IAA becomes cytotoxic only after HRP oxidation, but not prodrug, nor antibody-conjugate alone are toxic to normal mammalian cells. The fact that antibody-HRP conjugate is genetically engineered and produced in mammalian cell model is promising, as this kind of therapeutic molecule is more stable and less immunogenic.

The main challenge of my research was to characterize this newly designed monoclonal antibody and test whether this modification changed the functionality of trastuzumab-HRP. To check whether trastuzumab-HRP is able to recognize its antigen in denatured form, western blot and immunoprecipitation methods were used, and in native form, immunofluorescence method was used.

3. Experimental part

3.1 Materials and Methods

3.1.1. Cell lines.

To perform all the experiments described below the following cell lines were used: human breast adenocarcinoma cell lines - AU565 and MCF-7 ; and human non-small cell lung cancer cell lines - A549 H1299, H23 and grown at 37^{o}C in a 5% CO_2 humidified atmosphere. A549, H23, H1299 cell lines were kindly provided by colleague from DKFZ, Heidelberg. AU565 and MCF-7 cell lines were obtained from ATCC (American Type Culture Collection, Germany).

Eagle 's Minimum Essential Medium (EMEM) in Earle 's BSS and supplemented with 2mM L - glutamine, 1 % - nonessential amino acids was used as growth media for MCF-7 cell line, and Roswell Park Memorial Institute (RPMI) 1640 with 25mM HEPES and L-Glutamine were used as growth media for AU565, A549, H1299 and H23 cell lines. All media was supplemented with 10 % fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin.

3.1.2. SDS-electrophoresis and Western blotting.

All cell lines used in this research were seeded onto plates the day before the experiment and were used after reaching 70 % confluency. Cells were washed with 1×PBS. Next cells were trypsinized (1 ml of Trypsin/EDTA) and the trypsin inactivated with growth media containing serum (3 ml of growth media). Then cells were harvested by centrifuging at 1000 rpm, 5 min at room temperature. Cell viability and quantity were then measured by automated cell counter (Cell Countess, Invitrogen).

Next the cells were lyzed using 5×Laemmli sample buffer (10% SDS, 250mM TrisHCl pH 6.8, 0.1% bromophenol blue, 200 mM DTT, 50% glycerol), for 5 min at 100^{o}C and samples stored at -20^{o}C.

In this experiment, the same amount of $5x10^4$ cells were used for all the cell lines.

The samples were resolved by a 8% SDS-polyacrylamide gel and then transferred to PVDF-membrane using wet-transfer technique at 375 A for 2.5 hours in ice-cold 1×Towbin buffer (27 mM Tris, 190 mM Glycine, 7% EtOH). As the molecular mass marker *Page Ruler* Prestained Protein Marker (ThermoFisher Scientific, Lithuania) was used.

Protein binding sites were blocked on the PVDF-membrane by using non-block technique: air-drying for 15 min, 3 cycles of methanol-water hydration, incubation in methanol for 2 min and drying for 10 min at 37°C. Then the membrane was incubated with primary antibody solutions overnight on a shaker at 4°C. As primary antibodies 2 μg/ml Trastuzumab-HRP (designed and produced in Icosagen AS, Estonia) and 1:1000 α–ErbB-2 (Abcam, UK) were used. The dilutions of all used antibodies were prepared in 2% non-fat dry milk in TBST buffer (20mM Tris pH7.5, 160 mM NaCl, 0.1% Tween-20).

The filter was washed thoroughly 3 times for 15 minutes with TBST buffer *(*20mM Tris pH7.5, 160 mM NaCl, PBS 0.1 % Tween-20). PVDF membrane was then immersed in the solution of the secondary antibody in 2% non-fat dry milk TBST buffer and incubated on a shaker for 1 hour at room temperature. As secondary antibody we used 1:10 000 goat anti-human HRP antibody (for Trastuzumab-HRP) and 1:10 000 goat anti-rabbit-HRP antibody (for α-ErbB2). The membrane was washed again for 3 times for 15 minutes. Specific signals were visualized using the SuperSignal West Dura Extended Duration Substrate (ThermoFisher Scientific, USA).

3.1.3. Immunoprecipitation

All the cells were gown to 70% confluency prior to this study. The cells were washed with 1xPBS and collected using trypsin-EDTA and centrifuging at room temperature. The dry pellet was frozen at -80°C and the experiment was performed later. Next, the cells were lysed on ice with 2 ml of lysis buffer RIPA (50mM Tris pH7.4, 150mM NaCl, 2 mM EDTA, 0.1% NP-40, 0.1% SDS, 0.5%Na deoxycholate) for at least 15 min. Then, the lysates were centrifuged at max speed at 4°C for 8 min and the supernatants were carefully transferred into fresh tube.

Trastuzumab-HRP antibody was conjugated to magnetic beads according to manufacturer's protocol (Dynabeads Protein A, Life Technologies AS, Norway). The Dynabeads-Ab-conjugates were immediately used in immunoprecipitation of target antigen (ErbB2 protein) overnight at +4°C. Next, the Dynabeads-Ab-Ag complexes were washed, transferred to a new

tube, to avoid co-elution of proteins bound to the tube and denatured using 5xLaemmli buffer (10% SDS, 250mM TrisHCl pH 6.8, 0.1% bromophenol blue, 200 mM DTT, 50% glycerol) for 5 minutes at 100°C.

The samples were then loaded on a 8%-SDS-gel and resolved by electrophoresis. The signal was transferred to a PVDF-membrane using wet-transfer technique at 375A for 2.5 hours in ice-cold 1XTowbin buffer. As the molecular mass marker Protein Marker PM2600 (SMOBIO, Taiwan) was used.

Protein binding sites were blocked on the PVDF-membrane by using non-block technique. Then the membrane was incubated with primary antibody solutions (α-ErbB2 at final concentration of 1:1000) overnight on a shaker at 4°C. As secondary antibody goat anti-rabbit-HRP antibody was used at final concentration of 1:10000. The dilutions of all used antibodies were prepared in 2% non-fat dry milk in TBST buffer (20mM Tris pH7.5, 160 mM NaCl, PBS 0.1 % Tween-20). Specific signals were visualized using the SuperSignal West Dura Extended Duration Substrate (ThermoFisher Scientific, USA).

3.1.3. Immunofluorescence

The cell lines were seeded on 12-mm cover glasses in a 24 well plate at density of 1.5×10^5 cells per well the day before the experiment. After reaching 70-80% confluency, the live cells were incubated with primary antibody – Trastuzumab-HRP at 37ºC in a 5% CO_2 atmosphere 1 hour. Then, the primary antibody solution was removed, and the cells were washed three times with 1 mL of 1×PBS.

Next the cells were fixed with 4% paraformaldehyde in PBS solution for 15 min at room temperature. Then, the cells were washed 3 times with 1 mL of 0.05% PBS-Tw. To block the unspecific signal, cells were incubated with 1 ml of 2% BSA 0.05%-PBS-Tw for 1 hour at room temperature. Then, the cells were washed 3 times with 1 mL of 0.05% PBS-Tw.

The cell lines incubated with secondary antibody - anti-rabbit Alexa 488 for 1 hour at 37ºC. Then, the cells were washed 3 times with 1 mL of 2% BSA 0.05% PBS-Tw solution.

After air-drying the coverslips and they are mounted on a drop of antifade solution with DAPI and seal the coverslips with nail polish and visualize the target antigen by fluorescence microscopy.

3.2. Results.

3.2.1. Trastuzumab-HRP doesn't give a detectable specific signal on Western blot

Western blot with human breast cancer cell lines – AU565, MCF-7 and with human non-small cell lung cancer cell lines – A549, H1299 and H23 was performed using trastuzumab-HRP, to check whether this newly designed antibody is capable of ErbB2 protein detection from whole cell lysates using this method. The same number of cells was used for every cell line, so we could approximately quantify the amount of protein amount. The experiment was conducted several times, but the specific signal (at 185kDa) was not observed in desired quantity. Only the intermediate forms of ErbB2 protein, without post-translational modifications varying from 43-130 kDa, were detected.

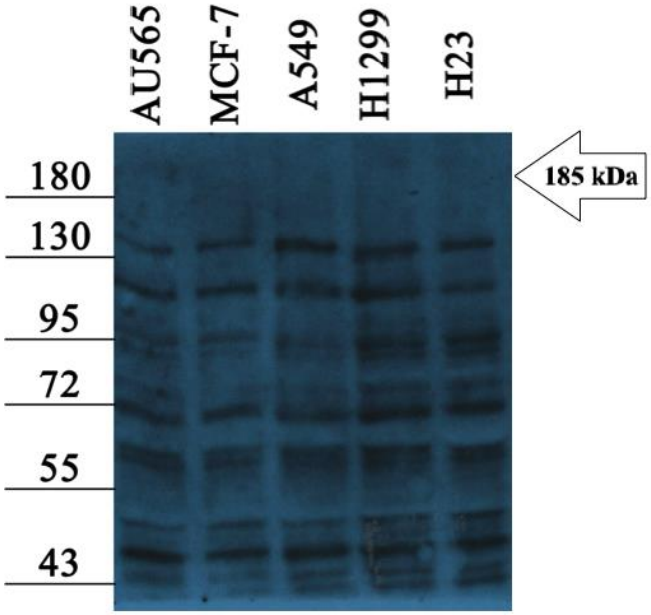

Figure 2. *Western blotting was performed with breast and lung cancer cell lines: AU565, MCF-7, A549, H1299 and H23. Trastuzumab-HRP, as the primary antibody in concentration 2 µg/ml and goat anti-human HRP, as secondary antibody in concentration 1:10 000 were used. Lysate from* $5x10^4$ *cells was resolved on each lane.*

3.2.2. Western blot with α –ErbB2

As WB with Trastuzumab-HRP did give positive results, this analysis was repeated with a commercial antibody α -ErbB2, to control whether the ErbB2 protein is present in the tested cell lines and confirm, that trastuzumab-HRP antibody is not suitable for detection of ErBb2

using Western Blot method. Here also, the same number of cells was used for every cell line, so we could approximately quantify the amount of protein amount.

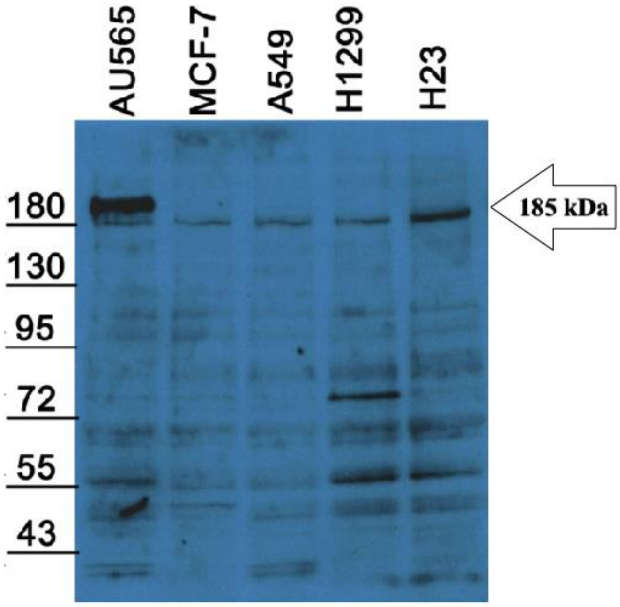

Figure 3. *Western blotting with breast and lung cancer cell lines: AU565, MCF-7, A549, H1299 and H23. The primary antibody α - ErbB2, in concentration of 1:1000, and secondary antibody goat anti-rabbit HRP, in concentration of 1:10 000, were used. Lysate from 5x10^4 cells was resolved on each lane.*

This experiment showed that there is ErbB2 protein present in every tested cell line. There are additional bands, because there are multiple forms of ErBb2 protein present in cells, that can have different stages of phosphorylation and glycosylation.

3.2.3. Immunoprecipitation

Since there wasn't any detectable signal in WB analysis with trastuzumab-HRP, it was decided to perform IP experiment, to enhance the signals and try again to check, whether the newly designed antibody trastuzumab-HRP binds to the ErBb2 protein. Similar number of cells was used for this experiment for every cell line.

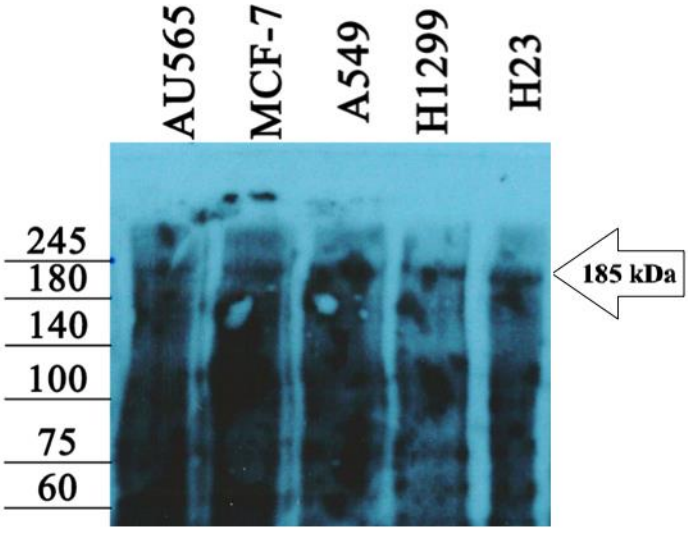

Figure 4. Immunoprecipitation *with breast and lung cancer cell lines: AU565, MCF-7, A549, H1299 and H23. Trastuzumab-HRP conjugated to magnetic beads was precipitated with antigen containing cell lysates and the signal was detected with α-ErbB2 and goat anti-rabbit HRP, as secondary antibody.*

Here we can see, that trastuzumab-HRP gives a specific signal, thus proving of being able to bind to ErbB2 protein.

3.2.4. Immunofluorescence

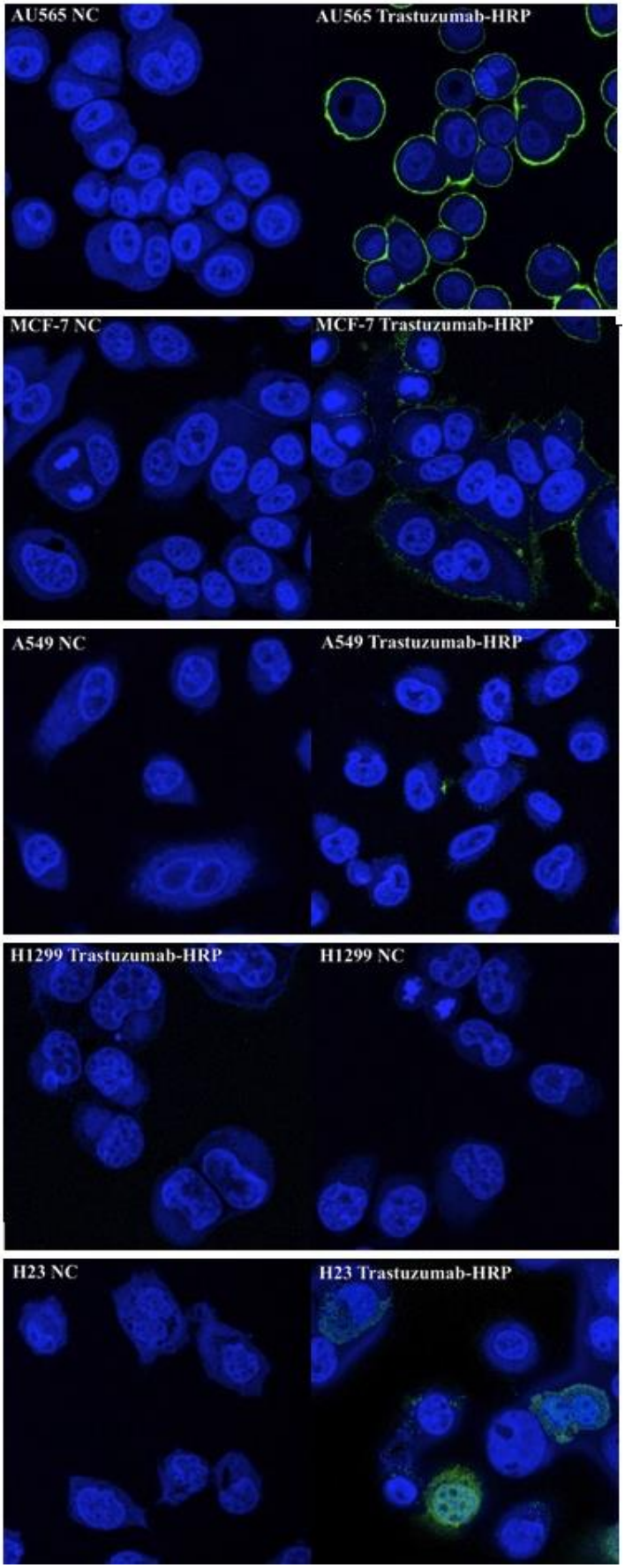

Figure 5. *Immunofluorescence microscopy with breast and lung cancer cell lines: AU565, MCF-7, A549, H1299 and H23. Trastuzumab-HRP, as the primary antibody, and anti-rabbit Alexa 488, as secondary antibody, were used. Cell lines incubated only with secondary antibody and DAPI were used as negative control (NC).*

The immunofluorescence was performed to check the presence of ErbB2 protein in human cell lines of breast and lung cancer – AU565, MCF-7, A549, H1299 and H23. The original protocol of immunofluorescent staining was modified, so that it would be possible to observe the signal of the trastuzumab-HRP that attached the live cells in the cell culture. So, the cells were fixed after the incubation of the live cells with trastuzumab-HRP, then the unspecific binding sites were blocked and then the cells were incubated with secondary antibody to visualize the signal. As a negative control, the same cell lines, incubated with secondary antibody and DAPI were used.

3.3. Discussion.

The field of cancer treatment has progressed steadily over the last decades and it is believed that more than 40% of deaths due to spreading of tumor could be prevented. The main challenge of targeting cancer is that there are hundreds of different types of cancer, which include broad range of gene mutations. As a consequence, as for now, there is no single kind of procedure available to provide effective universal treatment. In this respect, the medication includes chemotherapy, immunotherapy, hormone therapy and targeted therapy.

The prodrug-generating technology has developed a potential solution for the treating of cancer. It is performed by genetically designed antibody-HRP conjugates. The formation of adducts, together with strand breaks in DNA, is committing the cytotoxical role in tumor tissue. The result of this procedure is achieved through bystander effect in combination with high reproducibility and homogeneity of the antibody-HRP conjugates. This is attainable by using novel monoclonal antibody Trastuzumab-HRP conjugates.

The objective of our project was to learn the cell lines of breast cancer, because they contain the needed protein ErbB2, and also for the same reason we performed experiments on lung cancer cell lines as well. Considering the fact that the analysis of represented experiments by Western Blotting showed the Trastuzumab-HRP was absent on 185 kDa band, one might expect that the mentioned antibody does not bind to its antigen- ErBb2 protein. The given observation was checked with commercially available antibody α -ErbB2, our data revealed a clear sign of protein on needed band. The absence of ErBb2 on needed band can happen due to several reasons as low concentration of antibodies or antigen, prolong washing etc.

Consequently, the obtained results mean that the designed antibody-HRP conjugates cannot adequately detect the protein, but not due to missing adhesion. This conclusion was found by immunoprecipitation experiment that allows to examine higher number of material and provides an opportunity to detect the specific protein. It showed that Trastuzumab-HRP enables to find its target and then, captures the relative amount of the protein of interest. Hence, we have reached the conclusion that the antibody finds its antigen, making possible to use it in further developments of this treatment method.

But to determine whether our antibody can be used to detect needed protein in not only denaturated form, we finally checked it with immunofluorescence assay. The classical method reveals the presence of protein by special imaging techniques, but we modified the protocol so that we ensured our antibody to fix to its antigen before the next manipulation. This was

conducted because of our interest how the antibody acts in the native protein. It allowed us to see how the antibody conjugate works on live cells where the protein resides in natural form as in the reality, unlike in other experiments that took place in mutated cells where the protein was released in free form. The amount of signal differs in all cells. The results were obtained according to the review of literature. So, in the A549, MCF7, H12299, H23 the secondary antibody visualizes a few numbers of signal. But the association of AU565 cell lines with higher rate of ErbB2 that ranges 3 times more comparing to MCF7, for instance. (K. Subik et al, 2010)

Finally, our data support the idea that the Trastuzumab-HRP in concentration 2 μg/ml can be used in further development of ADEPT therapy in vivo in cell culture as single or additional targeting agent for breast and lung cancer patients.

SUMMARY

The acute problem of breast cancer treatment arose since the last century and it has attracted the attention of the researches to the need to elaborate the development of existing options.

The purpose of this graduation thesis *Characterization of antigen binding potential of a novel antibody-HRP conjugate designed for antibody-directed enzyme prodrug treatment of breast cancer cells* was to test the ability of the newly designed trastuzumab-HRP antibody-conjugate to bind its target antigen. The research was composed of three experiment, each of the method aimed at detecting of the binding of antibody-conjugate to its antigen ErbB2 protein, which according to literature, is expressed in human breast cancer cell lines. As it is known that some of the non-small cell lung cancers are also ErbB2 positive, some NSCLC cell lines were included in the analysis too.

Based on the western blotting assay results, it wasn't possible to detect sufficiently the designed antibody binding to its target antigen. But, results of the immunoprecipitation experiment showed that the trastuzumab-HRP is able to bind to ErbB2 in breast and lung cancer cells. Furthermore, using the alternative method, immunofluorescence, it was possible to detect the signal of trastuzumab-HRP binding to ErbB2 on live cells.

Based on the results gained from this research on characterizing new designed trastuzumab-HRP antibody-conjugate by testing its ability to bind its target antigen, it can be concluded that trastuzumab-HRP can be considered for further researches in ADEPT model *in vitro* on breast cancer and lung cancer cell lines.

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