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Combination antibody therapy of HER2 positive cancers

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Abstract

Focusing on anti-HER2 targeted antibody treatments, we examined the combination of trastuzumab and pertuzumab antibodies in the treatment of HER2-positive breast cancer. The phenomenon of trastuzumab resistance is of great importance due to its high, over 50% rate. In this work, we have demonstrated the superior efficacy of combination therapy and investigated its mechanism of action. To separately analyze antibody-dependent cell-mediated cytotoxicity (ADCC)-based and direct biological effects of the antibodies, we performed comparative studies of the whole antibodies and its F(ab’)2 fragments.

First, we aimed to find the most suitable nonradioactive method for quantitating in vitro ADCC against adherent cells. We compared FDA and CFSE release, propidium iodide uptake of the CFSE labeled cells, assays based on the detection of caspase activation in flow cytometry; and an impedance-based real-time cell adhesion test.

The largest dynamic range and the quantitation of antibody dose-response were possible with the latter, and therefore used we recommend this technique as the best non-radioactive choice for ADCC of adherent target cells, However, we recommend flow cytometric detection of caspase activation in target cells that are in suspension.

Next, we have produced intact functional F(ab’)2 fragments from both trastuzumab and pertuzumab by removing the Fc fragment using pepsin-agarose digestion. We have used these and the whole antibodies for in vitro and in vivo investigations. Neither the whole nor the F(ab’)2 antibodies decreased the proliferation of the in vitro trastuzumab-resistant JIMT-1 cells. The combination of both the whole or the F(ab’)2 antibodies showed an additive antiproliferative effect on the trastuzumab-sensitive BT-474 cells.

The F(ab’)2 fragments did not decrease the growth of JIMT-1 xenografts in vivo, but the whole antibodies were equally efficient. Combination of the whole antibodies decreased the proliferation further, which effect correlated with the decreased penetration of NK cells into the tumor, showed by ex vivo immunofluorescent labeling. In vitro, the combination of the two antibodies showed additivity in ADCC, but only at non-saturating concentrations.

Overall, we propose that the therapeutic antibody doses currently approved do not saturate ADCC in vivo. Thus it is advisable to use both antibodies at the maximal approved doses in combination as soon as possible after the diagnosis. As circulating tumor cells are pronouncedly sensitive to ADCC, combination therapy should be continued as metastasis prevention even when the primary tumor is resistant.
Introduction and literature review

Immunotherapy of cancer has been one of the greatest hopes of anticancer treatments. Oncologic protocols initially based on general cytotoxic chemotherapies and radiotherapies have started to move to cancer-specific treatment. Cancer immunotherapy was selected as “Breakthrough of the Year” by Science magazine in 2013. Currently the overwhelming majority of targeted cancer therapies approved for the clinic are still based on antibodies.

The development of cancer therapy received a new stimulus with the discoveries of the biological roots responsible for the emergence and progress of tumors and also revealed specific molecules that can be targeted by anti-cancer treatments. The aim of the targeted anti-cancer treatments is the killing of the tumor in situ by attacking the viability and proliferation of the cancer cells, without damaging healthy tissues.

By recognizing cell surface antigens, monoclonal antibodies evoke an antitumor immune response (antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity) and can also block the function of the recognized protein, i.e., a growth factor receptor thus resulting in a direct biological effect. The early successes of antibody therapies brought about even more interest and more antibodies were developed. The number of clinically approved monoclonal antibodies has been increasing exponentially.

Solid tumors form a closed niche by nature and are less accessible. The first successes were achieved on hematologic cancers, yet further development and optimization are needed for treating solid tumors.

Structure of antibodies (IgG)

Therapeutic antibodies are usually IgG class antibodies. The whole IgG (Wab, whole antibody) consists of three functionally different parts. Two identical Fab parts (“fragment antigen binding”) that recognize the antigen epitope, and the Fc part recognized by the immune cells’ receptors and complement proteins and evoking effector functions, i.a., degranulation of mast cells, basophil cells and eosinophils,
opsonization, and cell lysis through antibody-dependent cell-mediated cytotoxicity (ADCC).

By pepsin digestion, we can cleave the antibody in a way so that the two Fab regions remain connected by disulfide bonds forming an F(ab’)₂ fragment, and the Fc part is removed.

**Antibody-dependent cell-mediated cytotoxicity**

Natural killer (NK) cells are important effector cells of the cellular defense system. NK cells are activated through their FcγRIII receptors (CD16), binding immunoglobulins and recognize and destroy the cells opsonized by IgG (tumor cells or cells infected by a virus). This process is the antibody-dependent cell-mediated cytotoxicity.

Activation of NK cells requires intracellular signaling events, reorganization of the cytoskeleton and the cytoplasm membrane, and results in the release of granules containing perforin and granzyme B. Due to the higher pH and Ca²⁺ concentration, perforin binds to the membrane of the target cell and forms pores of 10-20 nm in diameter after oligomerization. Enzymes from the granules (including granzyme B) can enter the target cell through the newly formed pores and activate caspase cascade pathways leading to cell death. Due to the pores, the membrane will be permeable to water and ions which results in cell lysis. The process of membrane puncture and activation of the caspase cascade ending in the death of the target cell is called “lethal hit”.

**Quantitative methods for ADCC**

A widely accepted and used experimental method for cytotoxicity is the $^{51}$Cr release assay. However, this method requires the use of radioactive materials and poses a severe health risk. There has been a need in the literature for non-radioactive methods. We aimed to select the technique that makes it possible to detect slight changes and that alters the ADCC and the cells involved the least.
Release Assays

During release assays, we measure specific materials that are released from the target cells. These materials are either naturally in the cells, or the cells are filled with them initially. Cell death is evaluated by the amount of release. We may use fluorescent dyes instead of the $^{51}$Cr isotope for the labeling of the target cells, but the use of the own enzymes of the cell, i.e., GAPDH for measurement had been described as well. As the measured material is released only from those target cells with increased membrane permeability, by the amount released we can evaluate the death of the target cells due to treatment and in vitro ADCC.

Some authors describe the cytometric measurement of propidium iodide (PI) uptake to be more sensitive than release assays. PI enters the nucleus of those cells with increased permeability. The fluorescence intensity of PI increases upon intercalating with the DNA.

Quantitating ADCC specific caspase activity

Granzyme B released from the NK cell granules starts a caspase cascade in the target cell and thus contributes to the death of the target cell. In contrast to this, granzyme B activation is not seen during cell death due to aspecific stress; thus, ADCC can be evaluated by measuring caspase activation with fluorescent substrates.

Impedance-based cell analysis

Real-time impedance-based cell analyzers measure resistance and impedance between microelectrodes on the bottom surface of cell culture wells using alternating voltage. Cells attached to the bottom of the well change the properties of the circuit, and changes of the adherent cells are detected by the changes in the measured electric characteristics. The measured resistance depends on the coverage of the electrode by the cells, cell-cell connections and thus is useful for assessing cell proliferation, viability and cytotoxic effects.
The HER oncoprotein family

The members of the HER (ErbB) family (HER1-4) are type I transmembrane receptor tyrosine kinases. They have essential roles in cell proliferation, survival, and regulation of differentiation. They also play a key role in the pathology of several human malignancies.

HER1 (epidermal growth factor receptor, EGFR or ErbB1) is present on nearly all somatic cells. HER2 (ErbB2) has no ligand and is always active.

HER protein signaling

A unique and required step of the signaling of receptor tyrosine kinases is dimerization. The receptors pair up with similar receptors (homodimerization, i.e., HER1-HER1) or other receptors of the family (heterodimerization, i.e., HER1-HER2). The members of the dimers transphosphorylate each other which is the starting point of intracellular signaling pathways.

In the absence of a ligand, the extracellular domain of these receptors – except for HER2 - is in a closed conformation. In the event of ligand binding, the receptor turns into open conformation, and the dimerization arm becomes sterically accessible for association with other receptors. The dimerization arm of the HER2 is always in an active, accessible state. HER2 is thus the preferred dimerization partner for all HER family members. Autoactivation by homodimerization of HER2 may depend on its expression level. In human cancers, HER1-HER2 heterodimerization is also highly important.

Receptor tyrosine kinases are responsible for multiple signaling pathways, two of which are central in cancerous proliferation, metastasis and therapy resistance.

The RAS/MAPK pathway has a key role in cell cycle, migration, and differentiation. The Akt protein kinase is also essential in regulating metabolism, growth, proliferation, and survival. It is regarded as a proto-oncogene.
HER2 as an oncoprotein
Overexpression of the HER2 is important in the development and growth of several tumors. When activated, it drives proliferative and anti-apoptotic signals. HER2 is overexpressed in 25-30% of breast cancers and the overexpression is frequently due to gene amplification. As a result, the cancer will have an aggressive phenotype and bad prognosis: high proliferation rate of the cancer cells and metastasis formation, fast progression and resistance to chemo- and radiotherapy can be expected.

HER2 as a therapeutic target
The discovery that some anti-HER2 antibodies can inhibit the growth of HER2 overexpressing cells was a breakthrough and lead to the development of several antibodies against the extracellular domains of HER2 and EGFR. Of them, the anti-EGFR cetuximab (Erbitux®, 2004), the anti-HER2 trastuzumab (Herceptin®, 1998) and pertuzumab (Perjeta®, 2014) are clinically approved.

Trastuzumab (Herceptin®)
Trastuzumab, a humanized anti-HER2 monoclonal antibody, is the first clinically approved monoclonal antibody against solid tumors (1998). It is a first-line treatment for HER2 positive metastatic breast cancer patients. Being the first antibody with clinically proven efficiency, the mechanism of action of trastuzumab at the molecular and cellular level has been the subject of extensive research.

Trastuzumab can disrupt interactions between HER2 and other proteins and sterically block the proteolytic cleavage of HER2 resulting in diminished levels of the more active form of HER2. The juxtamembrane binding of trastuzumab can effectively evoke cross-linking, which facilitates endocytic mechanisms. Endocytosis and proteasomal degradation have been regarded as essential factors in HER2 regulation. Monovalent trastuzumab Fab fragments do not possess the same antiproliferative effect as the whole
antibody, which suggests that HER2 crosslinking is an important mechanism of action of trastuzumab. Although these effects of trastuzumab on signaling seem to be very important in vitro, there have been signs that in vivo the antibody-dependent cellular cytotoxicity might be even more important than the in vitro observed direct biological effects.

Despite the efficiency of trastuzumab, there is an innate or acquired resistance in about half of the cancers.

**Pertuzumab (Perjeta®)**

Pertuzumab (Perjeta®) is another humanized monoclonal antibody against HER2, which binds to the dimerization arm. It is more effective in disrupting HER3-HER2 complexes than trastuzumab. Despite these, the first clinical trials, under the trade name of Omnitarg®, failed.

**Combination of antibodies**

As trastuzumab and pertuzumab bind to distinct epitopes on HER2, we may use them simultaneously. Trastuzumab and another anti-HER2 antibody, L26, applied together in vivo, decreased the growth of N87 gastric cancer cells in a synergistic manner compared to monotherapy. Based on this preclinical animal experiment, the combination of anti-HER2 monoclonal antibodies might increase therapeutic efficiency; however, the underlying mechanism of action remains unknown. Extrapolating from the in vitro experiments, Friedman et al. suggested that antibody-mediated internalization may be the reason for the increased efficiency of the combined treatment. They have shown that this process becomes more efficient when the two antibodies bind to distinct epitopes as the consequent hypercrosslinking causing faster and more efficient internalization results in HER2 downregulation.

The phase III CLEOPATRA (CLinical Evaluation Of Pertuzumab And TRAstuzumab) study evaluated the efficacy and safety of pertuzumab in combination with trastuzumab
Final results of the trial showed that pertuzumab significantly increased the survival of HER2 positive metastatic breast cancer patients compared to pertuzumab substituted with placebo. In 2013, pertuzumab was approved for use in combination with trastuzumab and docetaxel as neoadjuvant treatment of patients with HER2-positive, locally advanced, inflammatory, or early-stage breast cancer in the United States. In the EU, approval was for the use in metastatic or locally recurring, non-removable breast cancer patients who had not received anti-HER2 therapy or chemotherapy previously. The ongoing APHINITY phase III trial continues to explore the benefits of pertuzumab + trastuzumab combination therapy against monotherapy after surgical removal for HER2 positive breast cancer patients. As the resistance against trastuzumab of HER2 overexpressing breast cancer is still a central question, exploring *in vitro* the mechanism of action of combination therapies may improve our understanding of their clinical potential and spectrum of applicability.
Aims

Data in the literature suggest that combination of antibodies against distinct epitopes of the same target may be beneficial both in vitro and in vivo. Thus, trastuzumab and pertuzumab antibodies against HER2 may be used in combination.

However, the mechanism of action of the combined use is not clear at the molecular and cellular level. Friedman et al. suggested that more effective internalization due to hypercrosslinking lies behind the combined effect. However, keeping in mind the central role of antibody-dependent cellular cytotoxicity in vivo, it may be possible that the two antibodies provide sterically complementary attachment sites for natural killer cells.

Based on these, we set the following objectives:

1. Find the most suitable and reliable nonradioactive method for quantitative analysis of in vitro ADCC against adherent cells.
2. In vitro and in vivo evaluation of the mechanism of action of the combined use of trastuzumab and pertuzumab.
3. Explore the clinical relevance of the combination therapy.
Materials and methods

Cell lines
Our target cell lines were HER2 overexpressing, adherent cell lines. For ADCC experiments we used freshly separated peripheral mononuclear cells and an immortalized NK cell line.

**SKBR-3** cells are one of the most frequently used and characterized cell lines in literature, *in vitro* moderately sensitive to trastuzumab. **BT-474** is another cell line from invasive ductal breast cancer, *in vitro* trastuzumab sensitive. **JIMT-1** is derived from the pleural metastasis of a clinically trastuzumab-resistant human breast cancer. These cells are *in vitro* trastuzumab-resistant and can be xenotransplanted into SCID mice, where it shows some initial trastuzumab sensitivity due to ADCC. **CD16.176V.NK-92** cells are from a human lymphoma of NK phenotype, stably transfected with the high affinity (176V) FcγRIIIA receptor (CD16). A variant cell line also coexpresses eGFP with CD16.

Flow cytometric methods
Cells were labeled in sorter tubes in 10 μg/ml antibody concentration for 10 minutes, then, if needed, labeled with secondary antibody in 10 μg/ml concentration after washing. After labeling, cells were washed twice and fixed in 4% buffered formaldehyde solution.
Cells were measured with BD FACScan, BD FACSCalibur or BD FACSaria III flow cytometers. Evaluation of the results was performed with FCS Express software (versions 3, 4 and 6).
Production of F(ab’)2 antibody fragments

F(ab’)2 fragments were produced from the whole IgGs by pepsin digestion. To separate the fragments, we used exclusion chromatography optimized for size and ionic strength (for trastuzumab Sephacryl S300, for pertuzumab Superdex 200). The absorbance of fractions was measured with a NanoDrop ND 1000 (Thermo Science), protein composition was assessed with non-reducing SDS-PAGE. F(ab’)2-s were tested for lack of the Fc, for preserved HER2 binding affinity and for inability to mediate ADCC.

Conjugation of antibodies with fluorescent dyes

Conjugation of trastuzumab and pertuzumab whole antibodies and their F(ab’)2 fragments with fluorescent dyes (Alexa Fluor 488 és Alexa Fluor 647) was done according to the manufacturer’s instructions.

Quality control of F(ab’)2 fragments

Assessment of HER2 binding affinity

HER2 binding affinity of the F(ab’)2 fragments was compared with the Wab parent antibody using direct immunofluorescent labeling. SKBR-3 cells were first labeled with Alexa Fluor 488 conjugated trastuzumab F(ab’)2 in 10x excess. Then the Alexa Fluor 647 conjugated corresponding whole antibody was added in saturating concentration (60 mM) and coincubated for 10 minutes. Labeling was performed in the opposite order, and also for pertuzumab. Cells were fixed in 1% PFA solution and measured with a flow cytometer.

Assessing the lack of the Fc part

Lack of the Fc part on F(ab’)2 fragments was assessed with indirect immunofluorescent labeling. SKBR-3 cells were labeled with Wab or F(ab’)2 antibody then washed with HEPES. We applied the Alexa Fluor 488 conjugated anti-human Fc antibody recognizing the Fc fragment or the Alexa Fluor 488 conjugated polyclonal anti-human (H + L) antibody recognizing the heavy and light chains as secondary
labeling. After washing, cells were fixed in 1% PFA solution and measured with a flow cytometer.

**In vitro cell proliferation**

*In vitro* proliferation was tested with an MTT-based colorimetric assay using the EZ4U kit. Cells were incubated with EZ4U for 2-4 hours, absorption of the metabolic substrate was measured on a Synergy HT Multi-Detection microplate Reader (Bio-Tek). Absorption was measured at \( \lambda = 488 \) nm, and corrected with background absorption at \( \lambda = 620 \) nm.

**Förster resonance energy transfer**

Analysis of disruption of EGFR-HER2 interaction was evaluated by using the acceptor photobleaching Förster resonance energy transfer (FRET) technique. The donor fluorophore of the anti-EGFR antibody is partially quenched by the acceptor fluorophore of the anti-HER2 antibody in case of close interaction (i.e., heterodimerization). Photobleaching of the acceptor relieves the donor from quenching, with a consequent increase of its fluorescence. From the increase, a FRET image can be created by the AccPbFRET software. Measurements were done with Zeiss LSM 510 confocal laser scanning microscope.

**In vitro ADCC assays**

*FDA and CFSE labeling*

For measurement of the release of fluorescent dyes we labeled freshly detached JIMT-1 cells with fluorescein diacetate (FDA) and carboxyfluorescein diacetate succinimidyl ester (CFSE). For fluorescent measurements, we used indicator-free DMEM.
**FDA and CFSE spontaneous release**

For examining spontaneous dye release, labeled JIMT-1 cells were seeded in 96-well plates for 24 hours. After gentle mixing of the medium, 100 µl sample was taken from each well and placed into wells of another 96-well plate for fluorescence measurement. To assess the remaining amount of dye in the cells, we changed the medium on the cells for fresh indicator-free medium and we lysed the cell with Triton X-100. To evaluate the time course of the spontaneous release, JIMT-1 cells were attached in 24-well plates, were labeled and washed, then 1 ml indicator free DMEM was placed on the cells. Hundred µl samples were taken from each well after gentle mixing of the supernatant at given time points. Fluorescence intensity of the samples was measured with a Synergy HT Multi-Detection Microplate Reader-rel (Bio-Tek) using 486/20 excitation and 538/20 emission filters.

**Flow cytometric ADCC assay based on PI uptake**

JIMT-1 cells were labeled with CFSE and resuspended in indicator-free medium. NK cells were added to the samples in NK-medium. Trastuzumab was administered at 10 µg/ml final concentration. Incubation was done at 37 °C for 5 hours and 100 µl samples were taken at given time points from all samples. PI was added to the samples and measured with a BD FACSCalibur flow cytometer after 5 minutes.

**PanToxiLux assay**

The PanToxiLux cytotoxicity assay detects granzyme B and caspase 8 activity in the target cells killed by NK cells. The target cells are distinguished from the NK cells by a TFL4 fluorescent label, and an NFL1 dye is used to label target cells already dead at the beginning of the experiment. Measurements were done with a BD FACSaria III flow cytometer.
**Impedance-based cell analyzer**

Measurements were performed on an ECIS ZΘ impedance-based real-time cell analyzer. JIMT-1 cells seeded in microtiter plates attached and spread on the bottom surface of the wells, covering the microelectrodes. After the resistance measured at 4000 Hz reached a plateau, treatments and NK cells were added to the wells. The measurement was continuously carried out until the resistance reached the minimal value and there was no more decrease (24-48 h).

**In vitro immune synapse formation**

JIMT-1 cells were grown on 2x9 well microslides. Trastuzumab and pertuzumab whole antibodies and F(ab’)2 fragments were added along with eGFP expressing NK-92 cells. CD16 was labeled with Alexa Fluor 647 conjugated anti-CD16 antibody, HER2 was labeled with Alexa Fluor 555 conjugated 76.5 Fab antibody.

**Xenograft tumors in SCID mice**

The effect of antibody treatment on JIMT-1 xenografts was examined *in vivo* in SCID (Severe Combined Immunodeficiency) mice. Due to the innate deficiency, these mice lack B and T cells, however, their NK cells are functional and recognize also human antibodies and generate ADCC.

SCID (C.B-17/Icr-Prkdcskid/IcrIcoCrl, Fox-Chase) mice were housed in the Animal House of the Life Science Building at University of Debrecen (registration number: III/4-KÁT/2015) in a specific-pathogen-free environment.

JIMT-1 cells were injected subcutaneously. Each mouse received a xenograft in both flanks. Antibody treatment was started immediately after xenotransplantation and was done intraperitoneally twice weekly. The injected dose was 100 µg (5 µg/body mass gram) of each antibody or F(ab’)2 fragment. Each treatment group consisted of 6-8 mice.
The experiments were done with the approval of the National Ethical Committee for Animal Research (# 4/2012/DE MÁB). All animal experiments were performed in accordance with FELASA guidelines and recommendations, and DIN EN ISO 9001 standards.

**Tumor xenograft sections and immunofluorescent labeling**

At termination, mice were dissected and the tumors were frozen immediately. 14 µm thick cryosections were made, air dried and stored frozen (-21°C).

For immunofluorescent labeling, we used anti-HER2 Alexa Fluor 488-76.5 Mab and Alexa Fluor 647-anti-CD45 Mab antibodies. Cell nuclei were stained with DAPI. Sections were mounted in Mowiol antifade.

**Confocal laser scanning microscopy**

Immunofluorescently labeled cells and sections were analyzed with a confocal laser scanning microscope (LSM 510, Carl Zeiss GmbH). For analysis of immune synapse between NK cells and target cells *in vitro*, 1.5 µm optical sections were used. For *ex vivo* tissue samples, 3 consecutive, 4 µm thick optical sections were taken covering the central 10 µm part of the sections.

**Statistical analysis of data**

Results of the experiment are presented with their mean/median ± SD or SEM. For dose-effect curves, data were fitted with the Hill equation. The dimerization inhibition effect of pertuzumab and pertuzumab F(ab’)2 was evaluated with one-way ANOVA with Holm-Sidak post hoc test, at α = 0.05. Variance analysis of in vivo tumor sizes at each sampling time point was done with one-way ANOVA with Tukey’s post hoc test, at α = 0.05.

*Ex vivo* analysis of the tumor penetration of the NK cells as a function to distance from the periphery was done with ‘one site competition’.

For statistical analysis, we used SigmaPlot 12 and GraphPad Prism 7 software.
Results and discussion

Finding the optimal non-radioactive in vitro ADCC method for adherent target cells

ADCC involves the interaction of at least two cells – target cell and NK cell. Hence, one highly important element of ADCC assays is the discrimination between these two cells during the measurement.

I.1. Fluorescent dye release assay

JIMT-1 cells were labeled with FDA or CFSE fluorescent dyes and the fluorescence of spontaneously released dye in the supernatant after 24 hours incubation was linearly proportional to cell number. CFSE was better kept intracellularly, the fluorescence intensity was smaller than FDA: However, the extent of spontaneous fluorescent dye release was so great in both cases that it obscured the ADCC-specific release after target cell death.

The spontaneous release of FDA and CFSE showed a saturating curve in time. Lysing the cells with detergent after saturation of spontaneous release did not increase the fluorescence intensity in the supernatant in the case of FDA and resulted in an only slight increase for CFSE. This increase, however, was not enough for detecting and evaluating slight changes in the efficiency of ADCC.

Hence FDA and CFSE release were not suitable in our system for assessing in vitro ADCC.

I.2. PI uptake combined with CFSE for flow cytometric ADCC measurement

The intracellular fluorescence of the more stable CFSE label can be sufficient for discriminating labeled target cells from non-labeled effector cells by flow cytometry. Detection of dead cells by PI uptake combined with CFSE label of the target cells could make it possible to separate four subgroups of the samples with mixed cells.
NK cells significantly decreased the survival of JIMT-1 cells compared to untreated control even without the presence of antibody. Adding trastuzumab to the system would not decrease the survival further. Changing the NK/target cell ratio or incubation time did not improve the evaluation of the difference between NK vs. NK + trastuzumab treatments.

Thus, we can conclude that PI/CFSE labeling for flow cytometric measurement is not specific enough to quantitate the effect of antibodies.

I.3. Flow cytometric measurement detecting intracellular processes specific for ADCC

One of the assays that detect the specific enzyme activities in the target cells after a lethal hit by an NK cell during ADCC is the PanToxiLux™ test which contains a substrate for both granzyme B and caspases that are activated by it. A TFL4 label is used to discriminate target cells from NK cells and is as efficient as CFSE in the flow cytometric measurement.

NK cells significantly increased the ratio of cells that received a lethal hit compared to untreated control, the survival decreased (p = 0.013). This was similar to that of the CFSE/PI method. Adding trastuzumab in addition to NK cells evoked more lethal hits, the survival of target cells significantly decreased compared to NK-only treated (p < 0.0001).

However, besides the NK + 10 µg/ml trastuzumab treatment, no other antibody or other antibody concentration showed a significant difference in survival compared to each other or the NK-only treatment.

In all the cytometric methods, target cells appear to suffer from being detached for a long time before and during the experiments.
I.4. Measurement of cell adhesion with impedance-based analyzer for ADCC

Real-time cell adhesion analysis showed that resistance values decrease in time during ADCC.

Dose-response experiments showed that differences in ADCC efficiency can be resolved in the 1-20 ng/ml range. However, doses of 100 ng/ml were already saturating ADCC.

End-points of the normalized cell index curves represent the ratio of the surviving target cells at the end of the experiment. Comparing the untreated, NK-only treated and NK + 10 ng/ml trastuzumab treated samples, the NK-only treatment did not decrease the survival significantly compared to untreated control (p = 0.34).

When all treatment groups were compared, trastuzumab and pertuzumab antibodies added over NK treatment significantly decreased survival at both 1 ng/ml and 10 ng/ml concentration (p < 0.01) compared to the NK-only treated sample. Significant differences were seen between different antibody concentrations as well (p < 0.02). Trastuzumab and pertuzumab antibodies were statistically similarly efficient at the same concentration; however, trastuzumab tended to be slightly more efficient in mediating ADCC.

Overall, we found that the impedance-based real-time cell analyzer can detect small, dose-dependent differences that the previous methods cannot. It can quantitate ADCC precisely, and NK cell background activity influences the final results to the least extent.

For these reasons, we chose the impedance-based cell analyzer for our further in vitro ADCC experiments.
II.

Evaluation of the mechanism of action and efficacy of anti-HER2 antibodies

Trastuzumab and pertuzumab have direct biological effects on most HER2 positive cells. To distinguish between the direct effect and ADCC we needed the antibody fragments that lack the Fc part and thus the mechanisms mediated by the Fc part. For our purposes F(ab’)2 fragments were required which, being bivalent fragments, are closer to the whole antibody in their direct biological effect (e.g., receptor crosslinking).

Preparation of F(ab’)2 fragments

As only the whole IgG trastuzumab and pertuzumab antibodies are available commercially, we had to prepare the F(ab’)2 fragments from the whole immunoglobulins in large amounts.

The Fc part was digested by pepsin immobilized to agarose beads. We optimized the digestion process; the optimal digestion time was 16 hours. The undigested or partially digested fractions were pooled and re-chromatographed after redigestion.

We verified that the F(ab’)2 fragments do not, but the whole antibodies do bind anti-Fc polyclonal antibodies. We compared the antigen recognition properties of the F(ab’)2 fragments to the whole antibodies with a competition assay. Trastuzumab F(ab’)2 administered first in 10x excess completely blocked the binding of trastuzumab Wab coincubated with the system for 10 minutes in saturating dose. Conversely, the Wab also blocked the binding of F(ab’)2-s. The results were similar with pertuzumab antibodies and F(ab’)2 fragments as well.

Assessment of in vitro proliferation

The effect of antibody treatment on in vitro cell proliferation during 3-days of incubation was assessed using an MTT-based colorimetric assay. Whole antibodies and
their F(ab’)\textsubscript{2} fragments did not decrease the proliferation of the known trastuzumab-resistant JIMT-1 cell line.

The proliferation of the trastuzumab-sensitive BT-474 cell line was inhibited to a similar extent by the trastuzumab Wab and F(ab’)\textsubscript{2}. Their EC\textsubscript{50} was not different. Pertuzumab IgG was less efficient compared to trastuzumab; however, its F(ab’)\textsubscript{2} fragment was much more effective with an EC\textsubscript{50} twenty times lower than that of pertuzumab.

Assessment of HER2 heterodimerization by FRET

The reason behind the increased efficiency of pertuzumab F(ab’)\textsubscript{2} may be the smaller size of the F(ab’)\textsubscript{2}, which better inhibits HER2 dimerization. For the assessment of this dimerization, we used acceptor photobleaching Förster resonance energy transfer. The EGFR-HER2 heterodimerization somewhat decreased upon pertuzumab treatment; however, pertuzumab F(ab’)\textsubscript{2} caused an even greater decrease.

Dimerization is inhibited by pertuzumab as it binds to the dimerization arm of HER2. The smaller size may provide better sterical condition, and thus the F(ab’)\textsubscript{2} can more effectively in inhibit dimerization and disrupt EGFR-HER2 dimers.

Effect of combination treatment on \textit{in vitro} proliferation

Combination of the whole antibodies or the F(ab’)\textsubscript{2} fragments did not decrease the proliferation of the intrinsically resistant JIMT-1 cells.

The combination was also examined using the trastuzumab-sensitive BT-474 cells as targets, and a complete isobole matrix was measured. Both in the case of the Wab and the F(ab’)\textsubscript{2} treatments, the combination treatment resulted in better growth inhibition than the maximal inhibition by single treatments. The isoboles ran straight between the two end-points (monotherapies); thus, the combined effect was considered additive, but not synergistic.
Assessing *in vivo* antitumor effects

The *in vivo* effects of antibody treatments were examined on JIMT-1 subcutaneous xenografts in SCID mice. Trastuzumab and pertuzumab whole antibodies decreased the growth of xenografts compared to HEPES treated control. The efficiency of the two antibodies did not differ. The combined use of the two antibodies – each at the same dose as in monotherapy – significantly decreased the tumor growth compared to monotherapy. However, as the tumor grew, and its extracellular matrix developed, a steric inhibition developed: after reaching ~250 µm tumor volume, the treated tumors started to grow at the same rate as the untreated ones.

In contrast, neither trastuzumab F(ab’)₂ nor pertuzumab F(ab’)₂, nor their combination decreased the growth of the in vitro resistant JIMT-1 tumors compared to untreated. We concluded that the Fc domains of the whole antibodies play a key role in the *in vivo* mechanism of action of the antibodies through evoking ADCC and this effect is at least additive for the dosage regimen used.

Histological evaluation of tumor sections

Ex vivo xenograft frozen sections were immunofluorescently stained to identify JIMT-1 cells why labeling human HER2, and NK cells by tagging mouse CD45. We examined the number of NK cells as the function of penetration depth and compared the treatments. The density of NK cells was higher in trastuzumab and pertuzumab treated tumors compared to untreated, and the combined administration of the two antibodies resulted in even higher NK cell density. In the combination treatment, NK cells could penetrate into the tumor deeper and developed larger and deeper fissures in the tumor tissue.

Not only the number of NK cells were greater in Wab treated and combined Wab treated tumors, but the NK cells were more active as well. Those NK cells that were in interaction with HER2 positive cells, probably forming a killing synapse, were flattened onto the surface of the tumor cells. Those NK cells distant from tumor cells were round
and not forming a synapse. The flat/round NK cell ratio was larger in combination treatment than in monotherapy, which also underlines the importance of antibody concentration for implementing efficient ADCC in antibody therapy.

**Evaluating ADCC in vitro**

We have verified the function of NK cells forming a cytotoxic synapse *in vitro* in the presence of saturating trastuzumab concentration using confocal microscopy. To evaluate the effect of combination therapy detected *in vivo*, we quantitatively assessed ADCC *in vitro*. For the experiments, we used JIMT-1 cells and NK cells expressing the high-affinity FcγRIII receptor. ADCC could be detected at 6.6 pM antibody concentration with the impedance-based cell analyzer. ADCC reached around 90% efficiency at 67 pM trastuzumab concentration. Antibodies did not cause the death of the target cells on their own, without NK cells.

Both trastuzumab and pertuzumab Wabs evoked ADCC in a dose-dependent manner and decreased the cell index. Pertuzumab was somewhat less efficient than trastuzumab. In combination treatments, if the sum of the two antibody concentrations (3.3 pM + 3.3 pM or 33 pM + 33 pM) were equal to the monotherapy concentration, cytotoxicity was the same. At non-saturating ranges, when the sum of the combined concentrations was twice that of the original monotherapy (6.6 pM + 6.6 pM), the extent of cytotoxicity was also doubled.

We determined the EC$_{50}$ values for each antibody using the Hill equation fitted to the data points. In combination, the EC$_{50}$ value was 6.1 pM, while in monotherapy it was 12.0 pM (trastuzumab) and 11.5 pM (pertuzumab). These values confirm the additive effect of the combination.

At saturating concentration (67 pM + 67 pM), the combination did not increase the efficacy.
Conclusions

One of our aims was to find and validate the ideal non-radioactive in vitro ADCC method for adherent target cells.

In release assays using either FDA or CFSE, the spontaneous release of the labeling material decreases the sensitivity and reproducibility of the experiment. Furthermore, the actual metabolic state of the target cells and the stress before and during the experiment can increase differences between results of separate experiments.

Propidium iodide frequently used to detect cell death was neither sensitive nor specific for cell death evoked by ADCC. The PanToxiLux and similar kits measuring caspase activity in target cells can detect the early steps of ADCC with better sensitivity and specificity. However, in these assays, we need to keep target cells in a detached state, which is not physiological for adherent cells and can alter cell viability, sensitivity and, importantly, the presence and targetability of the cell surface molecules. Fragmentation of the dead target cells and the increased spontaneous death due to the detached state can alter the result of the experiment.

Impedance-based measurements offer label-free real-time tracking of cell attachment, spreading, proliferation and cell death. Of all methods tried, ADCC measured with an impedance-based real-time cell analyzer offered the best sensitivity and was best quantifiable to the point of offering dose-effect resolution.

Thus, for measurement and quantitative examination of ADCC in vitro against adherent target cells, we recommend using an impedance-based cell analyzer. For non-adherent target cells, PanToxiLux and similar kits may be appropriate for the non-radioactive measurement of ADCC in vitro.

Next, we aimed to assess the efficacy of combination therapies by clarifying the underlying mechanisms. To distinguish between direct biological (signaling-based) effects of the antibodies and ADCC mediated by them in vivo, we compared the whole
trastuzumab and pertuzumab antibodies and their F(\text{ab}')\text{2} fragments. We have optimized pepsin digestion for the removal of the Fc domain of the antibodies.

Direct biological effect of the combination of trastuzumab and pertuzumab was quantitated on the \textit{in vitro} trastuzumab-sensitive BT-474 cells. Isoboles showed that combining the two antibodies had an additive effect for both the Wabs and the F(\text{ab}')\text{2} fragments. The maximal effect of the two antibodies was larger than that of either antibody alone. This confirms that the two antibodies influence HER2 signaling through different mechanisms, and these mechanisms can inhibit cell proliferation independently from each other.

Although the F(\text{ab}')\text{2} fragment of neither antibody nor their combination had a direct effect on the JIMT-1 cells, the whole antibodies could mediate ADCC both \textit{in vitro} and \textit{in vivo}.

Trastuzumab and pertuzumab had similar antitumor effects. Combination of the two antibodies was significantly more effective. \textit{In vivo} growth of the JIMT-1 xenograft was not inhibited by the F(\text{ab}')\text{2} fragments, they did not mediate ADCC.

Based on the \textit{in vivo} result, ADCC is a valuable mechanism of action of therapeutic antibodies which acts upon \textit{in vitro} trastuzumab-resistant HER2 positive tumors as well. The combination of the two antibodies increased NK cell penetration of the tumor and the number of NK cells attacking tumor cells. These effects were inversely correlated with the tumor size and progression.

The two antibodies \textit{in vitro} showed an additive effect when combined at non-saturating concentrations. At saturating concentration, when maximal ADCC was achieved for either antibody alone, the combination did not improve ADCC further. The explanation may be that ADCC is \textit{de facto} mediated the same way – through their Fc domain and the Fcγ receptors of NK cells – by both antibodies, and this mechanism is saturated. Since \textit{in vivo} combination therapy showed additivity the \textit{in vivo} doses administered probably do not saturate ADCC.
We have also observed that parallel to the tumor growth and development of an extensive extracellular matrix, the antibody-treated tumors started to grow at the same rate as the untreated controls. Thus, the antitumor effect of the antibodies on in vitro resistant JIMT-1 tumors decreases as the tumor grows, and their combination which improves the efficacy of ADCC can only prolong the progression-free period.

The combined use of trastuzumab and pertuzumab antibodies against HER2 positive cancers seemed to be safe and beneficial from the beginning and is currently being tested in the phase III APHINITY trial. From the results of the CLEOPATRA trial, the conclusion emerged that trastuzumab and pertuzumab therapy should not be stopped in metastatic breast cancers. This is a logical conclusion based given that circulating tumor cells can still be sensitive to ADCC.

Based on the additive effect of combination therapy on ADCC and due to the importance of inhibiting tumor dissemination, we suggest that combination antibody therapy be the first choice for adjuvant therapy of HER2 positive breast cancers, and we do not recommend interrupting or discontinuing treatment even upon local tumor progression. We also propose that pertuzumab in combination to trastuzumab should be given at maximal approved doses without any preselection criteria to achieve the greatest possible clinical benefit.
List of publications related to the dissertation

1. Tóth, G., Szőllősi, J., Vereb, G.: Quantitating ADCC against adherent cells: impedance-based detection is superior to release, membrane permeability, or caspase activation assays in resolving antibody dose response. 
   DOI: http://dx.doi.org/10.1002/cyto.a.23247 
   IF: 3.222 (2016)

   mAbs. 8 (7), 1361-1370, 2016. 
   DOI: http://dx.doi.org/10.1080/19420862.2016.1204503 
   IF: 4.881

   DOI: http://dx.doi.org/10.1016/j.cellsig.2015.11.012
   IF: 3.937

**Total IF of journals (all publications): 12.04**
**Total IF of journals (publications related to the dissertation): 8.103**

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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