SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

(PHD)

Identification of antitumor cell-mediated immune response modifying compounds by high-throughput screening

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UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN, 2023

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen

at 1pm, 6th of February, 2024

Theoretical background

Molecular alterations that lead to tumourigenesis

The maintenance of proliferative signaling is a major feature of tumor cells. Normal, healthy cells maintain a tight control of their cellular homeostasis, which includes the production and release of growth factors. Transformed cells, however, are able to evade this regulation through a number of alternative pathways. One such mechanism is to produce their own growth factor receptor ligands and thereby stimulate their reproduction in an autocrine manner. Furthermore, cancer cells can send signals to their healthy counterparts in the surrounding stroma to synthesise growth factors for them. However, it is known that by increasing the level or changing the structure of receptors on the cancer cell surface, they become hypersensitive to the sensing of growth factors and/or to the activation of signalling pathways without the presence of ligands.

In the sense of phenotypic plasticity, the cell adapts to constantly changing environmental conditions by changing its properties. However, tumour cells are able to switch off this function and become completely autonomous and independent of their environment. Epithelial-to-mesenchymal transition (EMT) refers to the change in cell phenotype from epithelial to mesenchymal.

Altering the metabolism is also critical for cancer cells to be able to provide the ATP energy, molecular building blocks needed for unbridled growth and division, and to do so in a short time. In addition, the cell has to defend itself against the damaging effects of oxidative stress and the transformed cell adapts to this at the metabolic level.

Defects in the intrinsic pathway of apoptosis have also been shown to contribute to unrestricted cell division. DNA mutations that confer a selective growth advantage to a cell are called "driver" mutations. However, "passanger" mutations do not enhance the proliferative capacity of the cell, but are found in the genome of the cancer cell. Loss-of-function mutations in protooncogenes (e.g. PIK3C, MYC, ERBB2) stimulate cell growth, division and survival, while loss-of-function mutations in tumour suppressor genes (e.g. BRCA1, BRCA2, TP53, PALB2) regulate DNA repair and cell cycle control. It is known that post-translational modifications (methylation, acetylation, ubiquitination, phosphorylation) at the N-terminal of histone proteins may also be involved in proliferation, differentiation or cell death processes through the mediation of histone methylase and demethylase enzymes. Furthermore, non-

coding microRNAs also have an impact on the process of tumourigenesis, as they may also act as oncogenes.

By forming new blood and lymphatic vessels, tumour cells ensure their own supply of nutrients and oxygen and the removal of their metabolic products.

One of the main obstacles to tumour progression is the overwhelming of the immune system, but transformed cells are able to bring the host defence system to their side, a phenomenon known as immunoediting. It is divided into three phases, which are, in a specific order, elimination, equilibrium and escape.

About breast cancers in general

Breast cancer is the most common cancer after lung cancer, and the most common among women. Breast cancer is considered a heterogeneous disease both at the molecular level and in terms of its response to therapy. The PAM50 (Prediction Analysis of Microarray 50) test classifies primary breast tumours into five molecular (intrinsic) subtypes based on the expression of 50 genes: basal-like, low claudin expression, high HER2 expression, luminal A and B. However, based on protein-level immunohistochemical grouping, we can talk about the so-called surrogate classification.

Therapies of breast cancers

The primary goal of all clinical therapies is to eliminate the life-threatening condition completely (curative therapy) or, if this is not an option, to improve or prolong the quality of life (palliative therapy). The removal of primary tumours has also changed considerably with the development of clinical medicine, as breast conservation surgery is now possible, as opposed to mastectomy, which is the removal of the entire breast. The so-called neoadjuvant therapy was first introduced in 1957 and was originally developed for the treatment of non-metastatic and inoperable breast cancer. It allows for a smaller intervention, which greatly speeds up the healing time and has a positive impact on the patient's psychological well-being, as it also provides a more aesthetically pleasing result. In contrast, adjuvant chemotherapeutic agents are given to the patient after the surgical procedure.

One group of cancer therapies is non-targeted radiotherapy. Radiotherapy indirectly induces single- or double-stranded DNA breaks in the genome, while radiolysis of water induces oxidative stress by generating reactive oxygen derivatives and indirectly destroys cells. The other group includes targeted therapies, hormone therapy and immunotherapy.

Immunotherapy

Monoclonal antibodies

One of the tools used in cancer therapies is the use of monoclonal antibodies. Monoclonal immunoglobulins are a collection of antibodies that recognise the same single epitope on the antigen. Their mechanism of action is direct elimination by acting as agonists or antagonists of cell surface receptors to induce tumour cell apoptosis. At the same time, cytotoxic agents (enzymes, toxin, radioisotope) can be conjugated to the antibody and thus result in the destruction of the target cell. The antibody molecule also has an opsonin function, i.e. it binds to the surface of the target cell and opsonises it, inducing immune cell activation and indirectly leading to cell death. Cell surface proteins carry epitopes for antibodies. Such proteins may include carcinoembryonic antigen (CEA), mesenchymal epithelial transcription factor (MET), epidermal growth factor receptor 1 (EGFR1 or ErbB 1), ErbB2 (HER2), ErbB3, vascular endothelial growth factor (VEGF), insulin-like growth factor 1 receptor (IGF1R), adenosine receptor, tumour necrosis factor (TNF)-coupled apoptosis-inducing ligand receptor 1 (TRAIL-R1), TRAIL-R2 and nuclear factor-kappa B ligand receptor activator (RANKL). HER2 and VEGF are involved in signaling pathways as tyrosine kinases. The monoclonal antibodies used in the clinic since 1997 are in humanised form so that the therapeutic agent itself does not trigger an immune reaction. This means that all fractions of the antibody, with the exception of CDR, are of human origin and produced by recombinant techniques. It is known from clinical data that the anti-HER2 antibody trastuzumab provides adequate therapy in only 35% of patients with HER2+ breast cancer.

Bispecific antibodies

Bispecific antibodies, in contrast to conventional antibodies, have two different antigen binding sites – two Fab arms – within a single molecule. They are capable of inducing ADCC, whereby one Fab region of the antibody recognises the tumour cell surface receptor and the other Fab region recognises the effector cell and acts as a bridge between them. However, it can be used as part of breast tumour therapy as an inhibitor of several signalling pathways. However, several studies have demonstrated that its efficacy in solid tumours is much lower than in hematological malignancy. Overexpression of TROP2, a protein that mediates cell growth processes, is predominantly a feature of triple-negative breast tumour cells (TNBC). A bispecific antibody can be used to achieve T cell-dependent elimination of TNBCs, as one of

the epitopes of the antibody is the T cell surface CD3 molecule and the other is the cell surface protein TROP2.

Cancer vaccines

A number of studies are investigating the efficacy and safety of dendritic cell, whole tumour cell lysate, RNA/DNA and peptide-targeted neoantigen vaccines in tumour therapy. Tumour-associated antigens are tumour antigens that also appear on the surface of normal, healthy cells, but in much lower amounts. Neoantigens, on the other hand, are tumour antigens that are the protein products of (non-synonymous) mutations affecting the amino acid coding sequence of tumour cells. Since these proteins are not expressed on normal cells, they are excellent therapeutic targets.

Adoptive cell therapy

In adoptive cell therapy (ACT), tumour-specific T lymphocytes, natural killer (NK) cells and dendritic cells are isolated from the patient, cultured, stimulated in vitro and then returned to the body. ACT focuses primarily on the isolation of cytotoxic CD8+ T lymphocytes, which have a cell killing function that enables them to eliminate targets immediately. Effector functions of CD8+ T cells include secretion of inflammatory cytokines (TNF-α, IFN-γ), expression of apoptosis-inducing proteins (FASL, TRAIL) and perforin-mediated cell lysis by releasing cytotoxic molecules. The specificity of ACT is achieved by returning autologous T cells whose T cell receptors have been genetically modified by CRISPR/Cas9. Based on the MHC (major histocompatibility complex) restriction, T cells only recognise peptide fragments that form a complex with MHC molecules. The efficacy of ACT can also be enhanced by improving the mechanism of recognition by T cells using lymphocytes expressing the chimeric antigen receptor (CAR). Thus a rapid and MHC-independent T-cell activation can be induced. In clinical trials for shotgun therapy, CAR T cells specific for the cell surface antigens ROR1 (receptor tyrosine kinase orphan receptor 1), NKG2D (natural killer group 2D) and MUC1 have been investigated.

Mechanism of trastuzumab

The human epidermal growth factor receptors

Human epidermal growth factor receptors with tyrosine kinase activity HER1 (ErbB-1), HER2 (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4). They are involved in the regulation of

cell growth, survival, adhesion, migration and differentiation signalling. All receptors contain a ligand (growth factor) binding extracellular domain (ECD), a transmembrane lipophilic segment and an intracellular tyrosine kinase domain (except HER3). Binding of growth factor to the extracellular domain induces dimerization of two receptors. If two identical receptors are paired, this is called homodimerization, whereas if two different receptors are paired, this is called heterodimerization. This process allows the trans-autophosphorylation of tyrosine side chains, providing a docking surface for downstream signalling downstream of Src homology-2 (SH2) or phosphotyrosine binding (PTB) domains.

HER2-positive breast cancers and Trastuzumab

Trastuzumab is a humanized anti-HER2 IgG1 monoclonal antibody that was approved by the FDA in 1998 for the treatment of HER2-positive metastatic breast cancer patients. The two antigen-binding sites of the antibody are specific to the juxtamembrane region of the extracellular domain of the HER2 receptor. Activation of the HER2 receptor-derived pathways is not only triggered by receptor dimerization but also by enzymatic cleavage of the HER2 monomer by metalloproteinase into an ECD, a transmembrane and a phosphorylated tyrosine kinase domain unit, the latter termed p95. Constitutive activation of the membrane-bound truncated receptor keeps the signal transduction on continuously. Trastuzumab is able to block the activation of the HER2 signalling pathway by several mechanisms. It can block the detachment of HER2 ECD through inhibition of metalloproteinase, and it can prevent receptor dimerization and trastuzumab-mediated receptor internalization and degradation. It is also capable of inducing ADCC by Fc receptor-bearing immune cells.

Trastuzumab-mediated ADCC

ADCC is a virus and tumour elimination reaction by the innate (natural) immune system. Its most characteristic effector is the natural killer (NK) cell, which recognises and lyses the target cell by antibodies specific to its surface receptors. In particular, the binding between the Fc region of the antibody that opsonises the target cell and the NK cell Fc receptors that recognise it creates the so-called immunological synapse (IS), which aims at the directed exocytosis of the effector cell towards the target cell. The synapse formation is a tightly regulated series of steps, resulting in a well-structured, highly stable, tight cell-cell contact. The cSMAC (central supramolecular activation cluster) contains the primary molecules that ensure the specificity of the interaction, while the pSMAC (peripheral SMAC) contains the LFA-1

integrin proteins. Once tight junction formation is complete, microtubule organizing center (MTOC) organization toward the IS is initiated in effector cells. The function of the MTOC is to provide a pathway for perforin- and granzyme-containing cytotoxic vesicles to move in a kinase- and dynein-dependent manner along microtubules towards the IS, where granules fuse with the plasma membrane. The perforins in the IS form large transmembrane pores providing a pathway for the granzymes to the cytosol of the target cell. Granzymes (granular enzymes) are cell death-inducing serine proteases, of which the most abundant are granzyme A (GzA) and B (GzB) types in NK cells. GzA induces caspase-independent cell death, whereas GzB induces apoptosis of the target cell via a 3,7 -dependent and -independent pathway.

Aims

Breast cancer is still the leading cause of death among women today. Several therapeutic approaches are available to treat the disease, such as anti-HER2 monoclonal antibody against HER2+ malignancy. Trastuzumab is able to paralyze tumor cell unlimited proliferation by several mechanisms, one of which is NK cell-dependent ADCC. However, we sought to answer whether this reproducible trastuzumab-based system could be enhanced by the action of other FDA-approved drug agents. To answer this, we aimed to perform the following experimental steps:

1. adjusting the appropriate ratio of the target and effector cells and the incubation time,
2. optimising an image analysis-based in vitro method for quantifying ADCC efficacy,
3. identification of compounds modulating ADCC efficacy by screening a molecular library,
4. validation of 'hit' compounds that modulate ADCC efficacy using techniques other than screening; and
5. characterisation of the mechanism of action of 'hit' compounds.

The discovery of ADCC dates back to 1965, so in the nearly six decades since its discovery, a great deal of detail has been revealed about the mechanism. In vitro, there are several techniques to choose from to get an idea of ADCC efficacy. However, a molecular biology method that can be used for high-throughput assays, i.e. testing a large number of compounds simultaneously, is not yet available. Therefore, we have sought to overcome the following challenges:

1. to set up an ADCC protocol that requires as little human intervention as possible, and
2. determining the extent of NK cell-mediated cytotoxicity using fluorescence microscopy.

Methods

Cell lines

All cell types were cultured in incubators at 37 °C, 95% humidity and 5% CO2. The mycoplasma-free status of the cells was checked regularly.

Electric-cell substrate impedance sensing (ECIS) measurement

In the first step of the ECIS experiments, the specially designed plates (8-well with 10 gold electrodes) were incubated with JIMT-1 medium (coating) for 1 h. This was followed by the addition of 105 JIMT-1 cells/well, as this cell number resulted in a continuous cell layer after 24 h, as indicated by the plateau phase of the exponential curve plotted from the impedance values. At this time, the ADCC reaction was initiated by adding trastuzumab antibody (10 µg/ml, humanized anti-HER-2 monoclonal antibody) and NK cells in different ratios (E:T=1:4, 1:2, 1:1 and 2:1). The same volume of JIMT-1 medium was added to the control wells without the presence of trastuzumab and NK cells. Impedance (I) values were recorded by the ECIS instrument at 4000 Hz and every 4 s. The extent of ADCC efficacy was inferred from the decrease in impedance values, which was directly proportional to the death of JIMT-1 cells. I used the following formulae for my calculations:

$$\text{Impedance reduction: } I_T / I_{T0},$$

the impedance of each well was plotted against time 0 of the ADCC experiment. I is the impedance value (ohms in SI units), T is the I value at that time.

$$\text{Relative ADCC efficiency} = \text{IADCC(T)} / \text{INO ADCC(T)}.$$

Identification of ADCC modulating agents by high-content screening (HCS)

JIMT-1 medium (50 µl/well) was added to 96-well HCS plates. 104 JIMT-1 cells were pipetted into each well in a volume of 75 µl. 24 h later, JIMT-1, SKBR-3, MDA-MB468-HER-2 and MKN-7 target cells were incubated with 0.5 µM Calcein-AM stain for 1 h at 37 °C. After washing with medium, cells were pre-treated (20 µM, 1 h, 37 °C) in 50 µL volumes with Enzo FDA Approved molecular library using the Freedom EVO liquid handling robot. We then started our ADCC experiment by pipetting 2x104 unstained NK cells and trastuzumab to JIMT-1. This was done by incubating the co-culture in 100 µl of JIMT-1 medium final volume, E:T=2:1, in the presence of 10 µg/ml trastuzumab or 2 µg/ml cetuximab (anti-EGFR antibody, Erbitux), in the presence of 10 µM concentration of drugs for 3 hours. Calcein stained JIMT-1

cells were imaged using Opera Phenix High-Content Analysis equipment at the beginning (0 h) and end (3 h) of ADCC (10x dry objective, 0.3 numerical aperture, non-confocal mode, ex: 488 nm; em: 500-550 nm). ADCC efficacy was determined using Harmony software with the following settings:

Calcein-stained JIMT-1 cells

The number of calcein-positive JIMT-1 cells in the focal plane was determined using the "Find cells" software module (channel: calcein; method: B; common threshold: 0.39; area: > 600 µm2; splitting coefficient: 20.4; individual threshold: 0.16; contrast: > 0.04). SKBR-3 and MDA-MB468-HER-2 cells were detected using the M-method (diameter: 40 µm; splitting sensitivity: 0.2; common threshold: 0.2). For MKN-7 cells, the M-method was the most accurate way to teach the software the location of the cells, allowing their identification (diameter: 40 µm; splitting sensitivity: 0.7; common threshold: 0.19).

JIMT-1 EGFP cells

The identification of EGFP-positive objects in the focal plane was set in the "Find cells" menu (channel: EGFP; method: M; minimum object diameter 80 µm). Where the software successfully identified EGFP+ JIMT-1 cells, but apparently not perfectly along the cell boundaries, we used the "Splitting sensitivity" (value 0.5) module to split a larger EGFP+ area into smaller units and also corrected the intensity of the background fluorescence for even more accurate image-based analysis.

The percentage of target cells surviving ADCC was determined using the following formula:

$$\text{Percentage of surviving cells (\%)} = 100 \times (\text{target cell count}_{3\text{hour}} / \text{target cell count}_{0\text{hour}})$$

Drug substances were considered as "hits" if they resulted in at least a 20% difference in ADCC efficacy.

LDH assay

JIMT-1 cells (4000 cells/well) were pipetted into 96-well plates and pretreated the next day with 20 µM sunitinib or an equal dilution of DMSO for 1 hour. Subsequently, 8000 NK cells were added to each well in JIMT-1 medium containing 10 µg/ml trastuzumab (5% FBS) and the co-culture was incubated at 37 °C for 6 hours. Then, the plate was centrifuged (250 g, 4 min) and the supernatant was pipetted into a new 96-well plate, followed by the addition of LDH reagent (supernatant:LDH reagent = 1:1 = 20 µl/well:20 µl/well) and after 20 min, absorbance was determined using a MultiskanTM FC Microplate photometer at 450 nm. The degree of cytotoxicity was calculated using the following formula:

$$\text{Cytotoxicity (\%)} = 100 \times (OD_{E+T+A} - OD_{E+T}) / (OD_{T\ lysate} - OD_{T}),$$

where "E" indicates effector cells, "T" indicates target cells, and "T lysed" indicates optical densitometry (OD) of JIMT-1 cells digested with lysis buffer, while "T" indicates trastuzumab antibody.

Cell morphometry analysis

Morphological changes in JIMT-1 cells were performed in a 96-well HCS plate with 4000 cells pipetted into each well. The next day, after treatment of the cells with sunitinib (20 µM, 1 h, followed by 10 µM, 3 h), they were incubated with 1000-fold diluted (5 µM) DRAQ5 fluorescent dye in JIMT-1 medium for 30 min at 37 °C, and images were captured without a wash step using an Opera Phenix High-Content microscope (20x objective; 0.4 numerical aperture; confocal mode). Harmony software was used for morphometric analysis. The cell number was determined using the "Find nuclei" option (channel: Alexa 633; method: B; common threshold: 0.15; area: > 50 µm2; splitting coefficient: 11.6; individual threshold: 0.35; contrast: >0.35), followed by the cell number using the "Find cytoplasm" command (channel: Alexa 633; method: A; individual threshold: 0.10). Next, in the "Select Population" menu (population: nuclei; method: common filters), the morphology parameters were listed using the software under "Calculate Morphology Properties".

Dispase assay

To assess the treatment-induced adhesion of JIMT-1 cells in a 24-well plate, 6x104 JIMT-1 cells were treated with 10 µM sunitinib malate or an equal dilution of DMSO for 3 h at 37 °C. After aspiration of the supernatant, the cells were washed with ice-cold PBS and 200 µl of 0.6 U/ml dispase enzyme was pipetted into the wells and incubated for 35 min at 37 °C. After removal of the enzyme, the settled cells were washed 5 times with 100 µl PBS and fixed with 10 % TCA solution (4 °C, overnight) for sulforhodamine B (SRB) measurement. The next day the plate was washed 1x with PBS solution, dried and incubated with 0.4 m/v % SRB stain for 10 min at room temperature. After incubation, a 1% acetic acid wash step, a drying step (with compressed air until the well was completely free of droplets, about 5 min), and dissolution of the bound dye in 1 mM Tris base solution and reading of the absorbance at 515 nm with a Tecan Spark multimode microplate instrument were performed.

Intracellular granzyme B staining

In a 96-well plate, 1x104 JIMT-1 cells were stained with 5 µM Cell Tracker Blue diluted in DMEM/F12 medium for 30 min at 37 °C. Then, cells were washed 2x with DMEM/F12 medium and pretreated with 20 µM sunitinib (50 µl/well) for 1 h. The ADCC experiment was started after pretreatment by adding 2x104 NK cells and 10 µg/ml trastuzumab JIMT-1 medium to target cells (sunitinib = 10 µM, 50 µl/well). The co-culture was incubated for 3 h, then collected by trypsin/EDTA digestion and washed with PBS. The stained JIMT-1 and unstained NK cells were then fixed in 4 % formalin (10 min, room temperature) and permeabilized with 0,1 % Triton X-100 solution (10 min, room temperature). Samples were blocked in 1 % BSA solution for 1 h and incubated with anti-granzyme B Alexa Fluor 647 antibody for 20 min at room temperature in the dark. The number of granzyme B-positive JIMT-1 cells was counted using a NovoCyte flow cytometer.

Analysis of cytokine secretion by ELISA (Enzyme-linked immunosorbent assay) method

$2x10^5$ CD16.176 V.NK-92 cells (10 µg/ml trastuzumab-free or -containing) in NK medium were incubated in a 96-well plate coated with 1 µg/ml HER-2-Fc for 24 h. The cells were also supplemented with sunitinib (0.01-30 µM). After one day of treatment, the concentration of interferon-gamma (IFN-γ) was measured by ELISA from the cell-free supernatant.

Cell cycle analysis

After triplicate separation of JIMT-1 cells, they were fixed in ice-cold 70% ethanol for 30 min at 4 °C. After washing the cells with PBS, they were incubated with 1 mg/ml RNase enzyme (30 min, 37 °C) and stained with 5 µM DRAQ5 stain for 30 min at room temperature. The amount of DNA was determined using a NovoCyte flow cytometer.

Western blot

JIMT-1 cells were collected in 150 µl of RIPA buffer supplemented with 1% PMSF and 1% PIC after a single PBS washing step. The cells were disrupted by sonication and the supernatant was collected by centrifugation. Protein concentration was determined by Direct Detect spectrophotometer. The protein sample was boiled in SDS sample buffer at 95 °C for 10 min and 20 µg of protein was separated by size on a denaturing polyacrylamide gel containing 12 % SDS (80 V, 15 min; 100 V, 70 min). The proteins were transferred to nitrocellulose

membrane (blotted; 400 mA, 90 min). The membranes were then blocked in 5 % skimmed milk powder (1 h, room temperature). Primary antibodies were diluted in 1 % skimmed milk powder and incubated overnight at 4 °C on a buffer. The next day, the membranes were washed in TBS buffer containing 0,1 % Tween20 (3x10 min, room temperature), and the HRP-conjugated secondary antibodies were diluted in 1 % skimmed milk powder and left to settle for 1 hour. For Akt detection, the pAkt antibody was removed by mild stripping, and the procedure was repeated with the anti-Akt antibody from the blocking step. After washing the membranes, the pre-development step was performed using an ECL-based Chemidoc Imaging instrument and quantified using ImageJ software.

Generation of EGFP (Enhanced Green Fluorescent Protein) expressing JIMT-1 cells

HEK293 cells with 80-90% confluence were transfected with the following four plasmids (10 µg, 1:1:1:1:1:1): pLP-1, pLP-2, pLP-VSV-G and pWOX -EGFP using Lipofectamine 3000 kit. The cell supernatant was replaced after 24 h and the lentivirus containing medium was collected 48 h post-transfection, filtered through a 0.45 µm filter and added to JIMT-1 cells with 8 µg/ml polybrene. Five days after viral transduction, EGFP expressing cells were sorted by FACS and further cultured or frozen.

Analysis of cell death in 3D JIMT-1 spheroid model

$2x10^3$/well of JIMT-1-EGFP cells were plated in a 96-well plate, precoated with 0.5% agarose solution to obtain a U-shaped cell surface and monitored continuously for 3 days for spheroid appearance (size, shape). The formed spheroids were transferred to glass-well Cell Carrier-Ultra 96-well plates previously incubated with 0.5 % Pluronic-F127 solution (45 min, room temperature). JIMT-1 spheroids were pre-treated with 20 µM sunitinib for 1 h, and then ADCC experiments were initiated by adding NK cells stained with 10 µM Cell Tracker Blue (E:T = 20:1) and trastuzumab antibody (10 µg/ml) (24 h, 37 °C). After 24 h, the co-culture was incubated with Annexin V-Alexa Fluor 647 fluorescent conjugate (1 h, 37 °C). Microscopic images were captured using an Opera Phenix confocal microscope (10x objective; 0.3 numerical aperture, confocal mode; EGFP: ex. 488 nm, em. 500-550 nm; Alexa647: ex. 640 nm, em. 650-760 nm). The images were analysed using Harmony software as described below. Spheroids were identified by EGFP fluorescence of JIMT-1 cells using the "Find texture" option and filtering by size (> 25 000). Objects at the edge of the field of view were removed from the analysis using the "Select population" menu. Annexin-staining (apoptotic) cells

appeared in the peripheral regions of the spheroids, so the intensity of annexin fluorescence in the apoptotic ring region was measured using the "Select region" (outer limit - 90%) module.

Intracellular LC3B staining

After treatment, JIMT-1 cells (1x104 cells/well) were washed with PBS and incubated with methanol at 20 °C for 30 min. After fixation, the cells were washed and permeabilized with 0.1% Triton X-100 solution (10 min, room temperature), later blocked in 1% BSA (1 h, room temperature). Cells were incubated with anti-LC3B antibody (500x 1% BSA) overnight at 4 °C. The next day, following the washing steps, cells were incubated with 1 μg/ml DAPI diluted in 1% BSA and secondary antibody (goat anti-rabbit IgG Alexa Fluor 647, 1000x) (1 hour, room temperature, covered). Microscopy was performed with an Opera Phenix confocal microscope (40x immersion objective; 1.1 numerical aperture; DAPI: ex. 405 nm, ex. 435-480 nm; Alexa647: ex. 647; em. 650-760 nm). The number of spots per cell was determined with the following settings using Harmony software. First, cells were identified by DAPI staining in the "Find nuclei" menu (channel: DAPI; mode C; common threshold: 0.2; area: > 60 μm2; splitting coefficient: 9.0; individual threshold: 0.2; contrast: 0.15; output population: nuclei). Using the "Filter image" option, the background intensity was reduced (channel: Alexa647; mode: Smoothing; filter: Gaussian; width: 10 px; output image: smoothed 647). The cell boundaries were identified using "Find cytoplasm" (channel: Smoothed 647; mode: F; membrane channel: Plane Map Alexa488; individual threshold: 0.05). Objects at the edge of the field of view were also removed using "Select population" (population: nuclei; mode: common filters with deletion of the border objects; region: cell). The spots were identified using the "Find spots" module (channel: Alexa647; ROI: cells [removed border objects] and cell; mode: C; radius: ≤ 4 px; contrast: > 0.18; uncorrected spot to region intensity: >1.7; distance: ≥ 1 px; spot peak radius: 0.5 px with calculate spot properties; output population: spots). Finally, the fluorescence intensity of the spots was determined using "Calculate intensity properties 1,2 and 3" (channel Alexa 647; population: cells (removed border objects), region: spots [1]; spot maxima [2] and spot borders [3]; mode: standard [1, 2, 3]; quantile fraction 50%).

HER2 immunostaining

In a 96-well HCS plate coated with JIMT-1 medium, 1x104/well JIMT-1 cells were pretreated with 20 μM sunitinib for 1 hour, diluted to a concentration of 10 μM with 10 μg/ml Alexa Fluor647-conjugated trastuzumab-containing medium and allowed to dilute for 3 hours.

After incubation, cells were washed in PBS and then fixed (4% formalin, 10 min, room temperature). In the permeabilization step - cells were allowed to stand in 100 % methanol at 20 °C for 10 min. Alcohol was removed, followed by blocking with 1 % BSA solution (1 hour, room temperature) and incubation with the primary anti-β-actin antibody (5000x, 1 hour, room temperature). Non-bound antibodies were removed by a wash step, after which cells were incubated with the secondary anti-mouse Alexa Fluor488 antibody (1000x) for 1 hour. After a further wash step, I labeled the nuclei with DAPI (1 μg/ml, 10 min, room temperature, covered) staining, which was also followed by a wash step. Images were acquired using an Opera Phenix HCS microscope (20x air objective; 0.4 numerical aperture, non-confocal mode).

Statistical analysis

Data are presented based on the results of at least three independent experiments (mean±SEM). For normality analysis, the Shapiro-Wilk test was used. Where data did not show a normal distribution, the Kruskal-Wallis test with Dunn's post-hoc test was used. Where the data were normally distributed, we used one-way ANOVA with Sidak's or Dunnett's post-hoc test, and in the case of two-way ANOVA, Tukey's post-hoc test. A value of $p<0.05$ was considered a significant difference. All statistical analyses were performed using GraphPad Prism 8.0.1 software (GraphPad Software Inc., San Diego, CA, USA).

Results

Setting ADCC parameters using the ECIS method

Based on our results, the effector - target cell ratios used (E : T = 1:4, 1:2, 1:1 and 2:1) resulted in different but significant ADCC efficacy. Furthermore, we found that it is sufficient to stay within the 3 h incubation time.

HCS assay optimisation for ADCC testing

Although ECIS is a reliable instrument with high sensitivity, the instrument at our institute is only suitable for 16 well experiments. Therefore, the experimental parameters used during ECIS were transferred to a microscope system optimized for high-throughput screening (HTS). ADCC efficacy was thus assessed by image-based analysis, where JIMT-1 cells were labeled with calcein-acetoxymethyl (calcein-AM) stain, while NK cells were added unstained to the co-culture. Statistical evaluation showed that a 2:1 E:T ratio resulted in a level of JIMT-1 cell death in 3 h that could be used to identify ADCC activators or inhibitors for screening.

Identification of ADCC potency reducing agents by high-content screening (HCS) essay

During the HCS essay, we incubated our ADCC co-culture with a molecular library of 774 druggable compounds. NK cells induced significant JIMT-1 cell death (mean of 56% cell death in 10 plates). Six components alone (without addition of NK cells) induced JIMT-1 cytotoxicity higher than 20%. In addition, none of the compounds tested enhanced ADCC efficacy compared to the DMSO solvent control, however, four compounds (hit; vincristine, colchicine, podophyllotoxin and sunitinib) provided at least 20% protection against ADCC.

Confirmation of the inhibitory effect of hits reducing ADCC efficacy by calcein staining

Confirmatory measurements with ADCC inhibitors were performed to verify the reliability of the screening. Based on our results, we can state that the inhibitory effect of all four pharmacological agents is reproducible (10 μM concentration).

Further development of the HCS-based ADCC essay using EGFP-expressing target cells

Nineteen different compounds – plus DMSO as a solvent control – were present in four replicates on the 96-well plate. The compounds included the three microtubule inhibitors

(colchicine, vincristine and podophyllotoxin) – as positive controls – for the assay, which were identified as ADCC inhibitors in our calcein-staining HCS experiments.

Transduced JIMT-1 cells can be clearly distinguished from non-fluorescent NK cells by their excitation at 488 nm wavelength. Based on our results, we conclude that NK cell-induced cell death (50% in the '+ NK group) can be reproducibly detected in our optimized system. Reproducibility is suggested by the re-identification of the three microtubule inhibitors previously identified as 'hit', however, none of the agents alone induced a cytotoxic response in the '– NK' group.

A common feature of the HCS experiments is that the confirmation of the effect of the compounds identified by screening has to be confirmed by a different technique.94 For this purpose, the standard method of ADCC quantification, ECIS, was chosen with the experimental parameters described previously. In all cases, a 2 : 1 E :T ratio resulted in JIMT-1 apoptosis of around 50%, which was markedly reduced in the presence of "hits".

Confirmation of the effect of sunitinib by LDH and ECIS

Based on our knowledge of microtubule disrupting agents, we decided to focus on studying the inhibitory effect of the multi-targeted tyrosine kinase inhibitor sunitinib. As a first step, we used a screening protocol to verify the reproducibility of the inhibitory effect of this agent. The measured data not only confirmed the inhibitory effect but also demonstrated a concentration-dependent ADCC efficacy. In addition to calcein staining, we also used LDH release assay and ECIS techniques to verify JIMT-1 cell resistance during ADCC reaction under sunitinib treatment. The enzyme lactate dehydrogenase is found in the cytoplasm of all our cells and exits the cell through the damaged membrane, so it can be detected from supernatants under experimental conditions in vitro. The LDH and impedance-based ECIS methods also clearly demonstrated a decrease in ADCC efficacy in the presence of sunitinib.

Sunitinib is a pan inhibitor of ADCC

To further investigate whether the effect of sunitinib was not limited to a single target cell line (JIMT-1), we performed trastuzumab-mediated ADCC in three additional HER2-positive tumour cell lines. The SKBR3 and MKN7 cell lines are endogenous, whereas the MDA-MB-468 cell line is exogenous HER2-positive. Target cell viability was measured by calcein staining and HCS microscopy. After evaluation of the data, we conclude that the effect of the sunitinib inhibitor is not JIMT-1 cell specific.

We also wanted to answer whether the previously observed inhibitory effect could be observed when ADCC is induced with anti-EGFR antibody, and therefore we also performed an in vitro experiment where we incubated a co-culture of JIMT-1 and NK cells with cetuximab antibody. The number of calcein-stained JIMT-1 cells was determined by fluorescence microscopy, the results of which allowed us to confirm that the inhibitory effect persisted.

Sunitinib inhibits NK cell activation

In the ADCC response, granzyme B is one of the cytotoxic molecules that is secreted into the immunological synapse by directed exocytosis from the granules of NK cells. We therefore investigated by flow cytometry whether sunitinib affects this release of granzyme B. Our measurements suggest that granzyme B enters JIMT-1 cells and that sunitinib reduced the proportion of granzyme B-positive JIMT-1 cells compared to the ADCC-DMSO control.

It is known that the interferon-γ (IFN-γ) cytokine secreted by NK cells induces a variety of immunological responses. Indeed, IFN-γ is responsible for inhibiting tumour cell proliferation or even initiating apoptotic processes. Therefore, the secretion of IFN-γ – as an activation marker – by NK cells was investigated by ELISA technique. ADCC was induced with trastuzumab-HER-2-Fc complex without the presence of the target JIMT-1 cell. The results of the colorimetric assay indicated that sunitinib inhibited the secretion of cytokine indicative of NK cell activation, which means that the inhibitory effect is consistent with our flow cytometric results.

We also performed an experiment in which only JIMT-1 or NK cell was treated and then ADCC co-culture was induced after a wash step. The efficiency of ADCC was monitored by calcein staining microscopy.

Sunitinib modifies the phenotype and adhesion of JIMT-1 cells

Since adhesion signaling may influence the regulation of cell death, it is suggested that sunitinib may also interfere with the ADCC response through this pathway. Therefore, we labeled sunitinib-treated JIMT-1 cells with Draq5 fluorescent dye and examined the morphological changes by HCS microscopy. DRAQ5 is a membrane-permeable fluorescent dye that binds stoichiometrically to double-stranded DNA, but also binds to RNA with much lower affinity, according to the manufacturer. Since the cytoplasm is rich in RNA, staining it allows the cell morphology to be examined under appropriate excitation. Our measurements revealed that sunitinib induces a significant increase in cell size.

Furthermore, we investigated how the adhesion of ADCC target cells changes upon sunitinib treatment. In this experiment, we examined JIMT-1 cells incubated with sunitinib using SRB assay coupled with dispase digestion. Our results show that the treatment resulted in markedly more JIMT-1 cells adhering to the plate surface.

Sunitinib treatment reduces cell surface HER-2 expression

The cell surface receptor HER2 is a key molecule in trastuzumab-dependent NK cell ADCC. It follows that the cell surface density of HER2 has a major impact on the efficacy of ADCC. Since the phenomenon of receptor recirculation is known,93,94,95,96,97 which involves the cell continuously "trafficking" its receptors between cell surface and cytoplasmic regions, we raised the possibility that sunitinib accelerates HER2 receptor internalization, thereby reducing the cell surface target of trastuzumab, which ultimately leads to escape from ADCC. To elucidate this, we studied HER2 localization in JIMT-1 cells by immunocytochemistry. In this experiment, JIMT-1 cells were incubated with a trastuzumab antibody conjugated to Alexa Fluor 647 dye, which revealed HER-2 expression heterogeneity in the cells, as microscopic images showed a marked decrease in cell surface receptor density. However, Western blotting confirmed that sunitinib had no effect on total HER-2 expression.

Sunitinib inhibits the cell cycle

HER2 is a member of the epidermal growth factor receptor family, which is the first player in the signal transduction pathway that controls cell division. When the number of HER2 receptors on the cell surface is reduced, it is predictable that the rate of cell proliferation is also reduced. Therefore, we investigated whether the concentration and incubation time of sunitinib we applied, resulted in any changes in the cell cycle. Following sunitinib treatment, the DNA content of JIMT-1 cells was assayed by RNase A enzyme followed by incubation with fluorescent dye labeling DRAQ5 double-stranded DNA using a flow cytometer. Our measurements revealed that the 4 h incubation time used during ADCC does not affect cell division. However, 24 h treatment already increases the number of JIMT-1 cells trapped in the G0/G1 phase, while decreasing the number of S phase tumor cells (4 h sun - 24 h sun).

Sunitinib induces autophagy in JIMT-1 cells

It is known from literature data that autophagy is a possible escape route from NK cell-mediated cell lysis. In fact, during ADCC, natural killer cell-derived granzyme b secreted into

the immunological synapse is degraded within the target cell via the autophagosomal pathway. We therefore investigated whether sunitinib induces autophagy in JIMT-1 cells. LC3B-II is a marker of autophagy that is generated by conjugation of the cytosolic LC3B-I and phosphatidylethanolamine and occupies the surface of nascent autophagosomes. The LC3B-II marker was examined by western blot technique, which showed that LC3B-II was increased in sunitinib-treated cells. Autophagosomes are also visible on immunocytochemical microscopy images, marked by LC3B-II dots. The analysis shows that the number of LC3B-II-positive objects is markedly increased by sunitinib treatment.

Sunitinib confers ADCC resistance in a 3D spheroid model

3D *in vitro* experiments are somewhat closer to the in vivo situation than 2D cellular experiments. Therefore, we generated EGFP-expressing JIMT-1 cells by transduction and transfection steps and used them to culture spheroids. Addition of NK cells to JIMT-1 spheroids resulted in tumor cell death, similar to 2D co-culture. The extent of cell death was determined by Annexin V staining from microscopic images. Apoptotic cells (Annexin V-positive) clustered in the peripheral region of the spheroids, forming a ring region. Treatment of the sephoroid with sunitinib reduced the appearance of Annexin V-positive cells in 3D slides.

Literature data support that integrins, in addition to their adhesion function, play an essential role in cell survival processes. Indeed, it is well known that inhibition of integrin signalling in breast cancer induces inhibition of apoptotic processes. Based on these data, we wondered whether inhibition of adhesion signaling would abolish sunitinib-induced resistance to ADCC response. The cell culture dishes were coated with Pluronic F-127 solution, which prevents adherent cells from settling. Our results showed that there is no difference between the efficacy of adherent and suspended ADCC, as the inhibitory property of sunitinib is still observed.

Sunitinib did not induce Akt phosphorylation in JIMT-1 cells

Receptor tyrosine kinases (RTKs) are key players in the tumorigenesis pathway by controlling cellular events of proliferation, survival and growth. Akt is a serine/threonine-specific protein kinase that mediates the RTK pathway. However, it is also known that Akt protein is also a central regulator of integrin-driven signal transduction. The activation of the protein is indicated by the appearance of its phosphorylated form, which was examined by

Western blot. Our results demonstrated that sunitinib treatment has no effect on Akt phosphorylation.

Summary

ADCC is one of the most prominent NK cell-mediated, trastuzumab-dependent therapy for HER2 positive breast cancer. My dissertation was based on the optimization and characterization of an *in vitro* ADCC model for screening molecular libraries. In addition, we also would have liked to better understand the mechanisms that influence ADCC.

By screening 774 FDA-approved compounds – a co-culture of unlabeled effector NK cells and calcein-stained JIMT-1 target cells – using the HCS technique, we identified four agents that resulted in a significant reduction in ADCC efficacy. However, cytotoxicity to JIMT-1 cells was observed for seven molecules. Colchicine, podophyllotoxin and vincristine sulphate known as microtubule inhibitors, which also explains the observed reduced JIMT-1 cell apoptosis. The inhibitors inhibit the formation of immunological synapses and also have a negative effect on intracellular vesicular transport processes, including exocytosis. Based on the literature, we focused on the fourth agent, which is the multi-target receptor tyrosine kinase inhibitor (RTKI) sunitinib. The cytoprotective effect of sunitinib on JIMT-1 cells was also demonstrated by LDH release and ECIS methods. The inhibitory effect of RTKI on ADCC was confirmed in additional HER2+ cell lines and in the presence of anti-HER1 antibody. This suggests that our observations are not limited to JIMT-1 cells or the HER2 receptor. NK cell function was assessed by their release of granzyme b and secretion of IFN-γ, and in both cases reduced activation was observed. Our experiments also highlighted a complex mechanism involving modulation of target cell phenotype, HER2 downregulation and autophagy induction. At the same time, sunitinib inhibited apoptosis of JIMT-1 cells in a 3D spheroid model and in suspension ADCC co-culture.

In an improved HCS assay for the identification of influencing ADCC agents, EGFP-transduced JIMT-1 cells were used. Our goal was to minimize human factors influencing ADCC and we automated almost all steps, including treatment and analysis steps.

In summary, we have identified the sunitinib as a new ADCC inhibitor, using a calcein staining method. The key message from our results is that FDA-approved sunitinib may interfere with trastuzumab-mediated ADCC therapy in patients with breast cancer. Moreover, we have developed an easier, faster and reliable new ADCC screening technique based on the detection of target cells that express EGFP.

Publikációs lista

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Registry number: DEENK/398/2023.PL
Subject: PhD Publication List

Candidate: Eliza Guti
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Guti, E.**, Bede, Á. M., Váróczy, C., Hegedűs, C., Demény, M. Á., Virág, L.: High-Content Screening Assay for the Identification of Antibody-Dependent Cellular Cytotoxicity Modifying Compounds.
   JoVE. 198, 1-12, 2023.
   DOI: http://dx.doi.org/10.3791/64485
   IF: 1.2 (2022)
2. **Guti, E.**, Regdon, Z., Sturniolo, I., Kiss, A., Kovács, K., Demény, M. Á., Szöőr, Á., Vereb, G., Szöllősi, J., Hegedűs, C., Polgár, Z., Virág, L.: The multitargeted receptor tyrosine kinase inhibitor sunitinib induces resistance of HER2 positive breast cancer cells to trastuzumab-mediated ADCC.
   Cancer Immunol. Immunother. 71 (9), 2151-2168, 2022.
   DOI: http://dx.doi.org/10.1007/s00262-022-03146-z
   IF: 5.8

Total IF of journals (all publications): 7
Total IF of journals (publications related to the dissertation): 7

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

29 August, 2023

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *