

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

**Investigation of receptor-oriented cancer therapy in human tumors  
overexpressing ErbB proteins**

by Tímea Erzsébet Váradi

Supervisor: Péter Nagy, Ph.D.



UNIVERSITY OF DEBRECEN  
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Members of the Examination Committee: Attila Bácsi, Ph.D.  
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The Examination takes place at Department of Physiology Medical and Health  
Science Center, University of Debrecen  
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## 1. Introduction

Despite an ever deeper understanding of the molecular background of malignancy and promising new therapeutic approaches cancer remains the leading cause of death in developed countries. Breast cancer is the second most common after lung cancer. In addition to rational drug development the natural habitat also provides a valuable source for drug discovery and development. Elisidepsin is a marine-derived anti-tumor agent with unique mechanism of action. We investigated the effect of the drug on the distribution of ErbB proteins and lipid rafts and compared the elisidepsin sensitivity of cell lines overexpressing ErbB receptors. In breast cancer ErbB2 is particularly important, since it is overexpressed in 20-30% of breast cancers. Trastuzumab (Herceptin®) was the first monoclonal antibody against ErbB2 developed to treat solid human tumors based on genomic research. The development of trastuzumab resistance is still unpredictable because its mechanism is unknown although a multitude of factors have been implicated. Masking of the trastuzumab binding epitope of ErbB2 by hyaluronan also leads to trastuzumab resistance. Such correlations have not been confirmed in human tumor samples.

The characterization of protein associations and their alterations induced by drug molecules not only reveals the intricacies of signal transduction networks but also promises to be a profitable tool for the development of efficient therapeutical approaches. In my PhD work I used both fluorescence resonance energy transfer (FRET) and proximity ligation assay (PLA) methods. The application of FRET is not widespread for histological specimens because the high background would introduce large errors into the calculations. PLA is an applicable technique for detection of protein complexes under harsh experimental conditions, but it has not been evaluated in a systematic way, prompting us to compare its performance to that of FRET quantitatively.

## 2. Background

### 2.1. *The epidermal growth factor receptor (EGFR) family*

The EGFR family of receptor tyrosine kinases comprises four members: ErbB1 (EGFR/Her1), ErbB2 (Her2/Neu), ErbB3 (Her3) and ErbB4 (Her4). These membrane receptors play an important role in early embryogenesis and development of the heart, the central and peripheral nervous system as well as in normal functioning of the mammary gland. Their mutation or overexpression can lead to cancer formation.

### 2.2. *Signal transduction activated by ErbB receptors*

Various ligands, growth factors can bind to the extracellular domain of ErbB proteins leading conformational changes and the induction of receptor homo- and heteroassociations. Depending on which ligand activated the receptor and exactly which tyrosine was phosphorylated different signaling pathways can be activated influencing cell proliferation, migration, differentiation and apoptosis. The ligands of ErbB proteins are synthesized as type I transmembrane proteins which can be proteolytically cleaved to release soluble growth factors. Both transmembrane and soluble ligands contain an EGF-like sequence which recognizes and binds to ErbB receptors. The ligands can be grouped based on their affinity to receptors.

**EGF-like ligands (e.g. EGF, TGF- $\alpha$ ):** they bind only to ErbB1 with high affinity.

**Heregulin (HRG, NRG-1):** is a neuregulin and has several isoforms. HRG- $\alpha$  and HRG- $\beta$  bind to ErbB3 and ErbB4 with different affinities. The more affine HRG- $\beta$  was used in my experiments.

### 2.3. *The role of ErbB proteins in breast cancer, molecular tumor therapy*

ErbB proteins are at the forefront of interest in rational drug development. Overexpression of ErbB2, a family member lacking a soluble ligand, has been strongly linked to poor prognosis in breast cancer. ErbB2 is a therapeutic target in

the treatment of breast cancer since in addition to serving as a prognostic factor it is targeted by low molecular weight tyrosine kinase inhibitors and monoclonal antibodies.

Trastuzumab (Herceptin®): it was the first monoclonal antibody against the extracellular part of ErbB2 developed for the treatment of solid tumors based on genomic research. Trastuzumab is the humanized version of the original mouse-derived monoclonal antibody designated 4D5. The anti-tumor effect of trastuzumab was clearly demonstrated in phase II and III clinical trials, but its exact mechanism of action is still unknown.

Pertuzumab (Omnitarg®): it is a humanized monoclonal antibody against the extracellular part of ErbB2 binding to domain II. Thus, it sterically inhibits heterodimerization of ErbB2 even if ErbB2 is not overexpressed.

Masking of the trastuzumab binding epitope of ErbB2 by MUC4 or hyaluronan has been shown to lead to trastuzumab resistance.

#### ***2.4. Mechanism of action of trastuzumab***

Trastuzumab combined with chemotherapy is an efficient and well-tolerated therapy. The mechanism of action of the antibody is thought to involve antibody- or complement-mediated cytotoxicity and direct inhibitory effects on the ErbB2-overexpressing cells. The latter includes diminished signaling through ErbB2 and Src and subsequent activation of the PTEN phosphatase, disruption of the ErbB2-ErbB3 complex resulting in inhibition of Akt-mediated signaling. The relatively minor side-effects of trastuzumab are also thought to be based on the inhibition of ErbB-mediated signaling in the heart.

### **2.5. Trastuzumab resistance**

Although the application of trastuzumab in combination with other drugs prevents the accurate assessment of the development of resistance to the antibody itself, the decline in responsiveness during long-term treatment is very common. A multitude of factors have been implicated in trastuzumab resistance including loss of PTEN expression, activating mutations in the catalytic subunit of PI3K, increased signaling through other ErbB proteins, Met or IGF1R, overexpression of calpain-1, expression of a constitutively dimerized, truncated form of ErbB2 by alternative initiation of translation. We have shown previously that masking of the trastuzumab binding epitope of ErbB2 by MUC4 or hyaluronan also leads to trastuzumab resistance. In particular, the role of hyaluronan was established by showing that inhibition of its synthesis led to increased binding and anti-proliferative effect of trastuzumab *in vitro* and in mouse xenograft experiments. In my PhD work we investigated this correlation in human tumor samples.

### **2.6. The role of hyaluronan in tumor progression and in ErbB2 masking**

Hyaluronan plays important roles in carcinogenesis. In tumors it is produced by hyaluronan synthases (HAS1-3) in the malignant cells themselves or in stromal cells. Hyaluronan provides a supportive matrix for cellular growth and motility, but it also plays a more active role by stimulating proliferation, migration, angiogenesis, multidrug resistance and epithelial-mesenchymal transition. The latter responses are induced by binding of hyaluronan or its low molecular weight degradation products to cell surface receptors (CD44, RHAMM). Hyaluronan seems to play a double-faced role in malignancy. Adenocarcinomas with high hyaluronan content tend to grow aggressively and are poorly differentiated, while in squamous cell carcinomas reduced hyaluronan content is associated with a better prognosis. In particular, overproduction of hyaluronan in mammary carcinoma accelerates tumor growth

through the recruitment of stromal cells, stimulating angiogenesis and is associated with poor prognosis.

Although the role of hyaluronan has been thoroughly investigated in human tumors we have undertaken the experiments presented in my PhD work to establish if it is involved in inhibiting trastuzumab binding to ErbB2 in human breast cancer *in vivo* as expected based on our previous *in vitro* and xenograft experiments.

### ***2.7. Molecular mechanism of action of elisidepsin***

Kahalalide F has been isolated from *Elysia rufescens*, an indopacific mollusc acquiring and accumulating it from algae (*Bryopsis pennata*) on which the *Elysia* mollusc feeds. Due to the scarcity of the natural source elisidepsin (Irvalec) with a closely related structure has been synthesized which is currently undergoing phase II clinical investigations. It has been observed that Kahalalide F induces the disruption of lysosomal membranes, nuclear fragmentation and necrotic cell death. It was also suggested that Kahalalide F and elisidepsin act by inhibiting Akt activity. A recent paper about elisidepsin reported that *S. cerevisiae* lines mutated in genes involved in the regulation of vesicular trafficking were the most sensitive to the compound. RNA interference-mediated knockdown of fatty acid 2-hydroxylase (FA2H) expression increased resistance to elisidepsin suggesting that the enzyme plays a role in the mechanism of action of the drug. Fatty acid 2-hydroxylation has been implicated in hydrogen bond formation and stabilization of lipid rafts. To investigate whether ErbB proteins play a role in determining elisidepsin sensitivity, we analyzed the importance of ErbB1-3 in elisidepsin-induced responses.

### ***2.8. The role and examination of molecular associations***

The association of growth factor receptors is the first step in signaling processes. A large variety of methods is available for the investigation of the protein

interactome, but some of them have disadvantages. Widely used methods are listed below:

1. Qualitative-semiquantitative methods:

➤ conventional molecular biological techniques („*co-capping*”, *immunoprecipitation*, *chemical cross-linking*, *Western-blot*): they provide valuable information about the localization of proteins, but the applied isolation processes prevent the examination of proteins in their natural environment.

➤ yeast two-hybrid (*Y2H*) and bimolecular fluorescence complementation (*BiFC*) techniques: they are normally used for detection of protein complexes and protein interactions, however the reconstruction of transcription factors or fluorophores is irreversible. Despite of the aforementioned disadvantages they can be used in high throughput screening (*HTS*) techniques which are indispensable methods in the early phases of drug development.

2. Quantitative methods:

➤ colocalization by *microscopy*: it is defined by the presence of two or more different molecules residing at the same physical location in a specimen. Within the context of a tissue section viewed in the microscope, colocalization may indicate that the molecules are attached to the same receptor, while in the context of digital imaging; the term refers to colors emitted by fluorescent molecules sharing the same pixel in the image.

➤ fluorescence resonance energy transfer (*FRET*) and fluorescence correlation spectroscopy (*FSC*): they are based on accurately described physical principles and offer quantitative insight into protein clustering but their widespread application for specimens with high fluorescence background (e.g. tissue sections) is hampered by their sensitivity to errors in background subtraction.

Therefore, efforts have been made to develop techniques applicable under conditions of high background as well. Proximity ligation assay (*PLA*) is a novel method for the

detection of protein complexes under harsh experimental conditions. It is based on the amplification of a DNA sequence whose generation is dependent on the molecular proximity of two antibodies labeled by different oligonucleotides (priming and non-priming), which will form a circular DNA molecule with the help of connector oligonucleotides. The amplified DNA sequence is detected by a fluorescently labeled probe. Due to the amplification of the signal by the rolling circle amplification (RCA) reaction and the requirement for the proximity of two antibodies, the PLA technique is both sensitive and specific and provides a high signal to noise ratio. The protocol of *in situ* PLA contains the following steps: blocking, conjugation of PLA probes, hybridization, ligation, amplification and detection.

### ***2.9. The structure and biological function of lipid rafts***

The plasma membrane of cells contains special microdomains termed lipid raft composed of a mixture of glycosphingolipids and protein receptors. Lipid rafts are more ordered and tightly packed than the surrounding bilayer, but diffuse freely in the membrane bilayer. One key difference between lipid rafts and the plasma membranes from which they are derived is lipid composition. Research has shown that lipid rafts generally contain 3 to 5-fold the amount of cholesterol found in the surrounding bilayer. Also, lipid rafts are enriched in sphingolipids such as sphingomyelin. Because of their detergent resistance, lipid rafts are also called detergent-insoluble glycolipid-enriched complexes. A typical example for membrane proteins found in lipid rafts is the GPI-anchored proteins. They are organized into high density clusters of 4–5 nm radius, each consisting of a few molecules and different GPI-anchored proteins.

### **3. Materials and methods**

#### ***3.1. Cell lines and plasmids***

The experiments were performed on SKBR-3 (trastuzumab-sensitive human breast tumor), CHO (chinese hamster ovary) and A431 (human epithelial carcinoma) cell lines. CHO cells stable transfected with ErbB2 (CHO-ErbB2), or with ErbB2 and ErbB3 (CHO-ErbB2-3) were produced earlier produced in our department. The A431-erbB1-eGFP, A431-erbB2-mYFP and A431-erbB3-citrine cell lines stably express ErbB1-eGFP, ErbB2-mYFP and ErbB3-citrine.

#### ***3.2. Measurement of cell viability***

The short-term cytotoxic effect of elisidepsin was tested by microfluorometric PI uptake assay. Cells were seeded at high density in black, clear bottom 96-well microtiter plates and allowed to grow to confluency. Fresh culture medium in the absence or presence of different concentrations of elisidepsin was added in quadruplicates and the uptake of PI was quantified by plate fluorimetry at excitation and emission wavelengths of 531 and 632 nm, respectively, at 37 °C using a Victor3 Multilabel Counter. The long-term effect of elisidepsin on cell viability was assayed by measuring the oxidation of a water-soluble tetrazolium salt by mitochondrial dehydrogenases using the cell proliferation reagent WST-1. Cells ( $7 \times 10^3$ ) were plated into single wells of 96-well plates 24 h before the experiment. Cells were treated with a dilution series of elisidepsin for 2 h in triplicates followed by incubation for 72 hours in cell culture medium in a CO<sub>2</sub> incubator at 37 °C. The absorbance of the WST-1 reagent was measured by an ELISA reader at 450 nm and 620 nm. The IC<sub>50</sub> value, the concentration leading to the death of 50% of the cells, was determined.

### ***3.3. Fluorescence resonance energy transfer (FRET)***

In FRET energy is transferred in a nonradiative fashion from an excited donor molecule to a nearby acceptor. FRET efficiency depends on the distance between the donor and the acceptor therefore it can be used to detect protein- protein interactions. FRET was measured with a FacsArray flow cytometer. Antibodies labeled with AlexaFluor546 and AlexaFluor647 were used as donor and acceptor, respectively. The donor, FRET and acceptor fluorescence intensities were measured in the Yellow, Far Red and Red channels, respectively. The Yellow and Far Red intensities were excited with a 532 nm solid state laser and detected using a 585/42 nm bandpass and a 685 nm longpass filter, respectively. The Red intensity was excited at 635 nm using a diode laser and measured using a 661/16 nm bandpass filter. The FRET efficiency was calculated on a cell-by-cell basis using the ReFlex software. Calculation of FRET intensity ( $I_{FRET}$ ) was often performed in PLA experiments in addition to determining the FRET efficiency.

### ***3.4. Confocal microscopy***

A Zeiss LSM510 confocal laser scanning microscope was used to image fluorescently stained cells. Confocal stacks were acquired with the pinhole size adjusted to 1 Airy unit and image distances of 0.5  $\mu\text{m}$  along the Z axis using a 63 $\times$  (NA=1.4) oil immersion objective. Image analysis and the preparation of orthogonal projections were carried out with a custom-written Matlab program incorporating DipImage commands.

### ***3.5. Measurement of fluorescence anisotropy and generalized polarization***

Trypsinized cells were resuspended in Hank's buffer at a concentration of  $10^7$ /ml and labeled with 2  $\mu\text{M}$  TMA-DPH or 2.5  $\mu\text{M}$  Laurdan at room temperature for 20 min. After TMA-DPH labeling cells were diluted in Hank's buffer without

washing to a concentration of  $10^6$ /ml for fluorescence anisotropy measurements, whereas Laurdan-labeled cells were washed once and resuspended at a concentration of  $10^6$ /ml in Hank's buffer. Fluorescence measurements were carried out with a Fluorolog-3 spectrofluorimeter. TMA-DPH was excited at 352 nm and its emission was measured at 430 nm. The fluorescence anisotropy ( $r$ ) of TMA-DPH was measured. Laurdan was excited at 350 nm and its emission was detected in the blue range of its emission spectrum at 435 nm ( $I_{blue}$ ) and at the red edge at 500 nm ( $I_{red}$ ). Generalized polarization (GP) of Laurdan fluorescence was calculated.

### ***3.6. Patients, tissue samples***

Patients diagnosed with ErbB2-overexpressing breast cancer were recruited into the study. Samples for research purposes were collected during surgery concomitantly with tissue samples taken for histopathological examination. ErbB2 overexpression was established by immunohistochemistry using the 4B5 rabbit anti-ErbB2 monoclonal antibody. ErbB2 amplification was analyzed by FISH. Only patients showing strong positivity (2+ or 3+) in both tests were involved in the project. Patient follow-up data were collected after surgery by regular outpatient visits. The samples of 45 patients were stained. On average ~15 images were taken and analyzed from every patient.

### ***3.7. Staining of tissue samples and confocal microscopy***

Tissue sections were fixed in 4% formaldehyde followed by a two-step blocking procedure. First with BSA (bovine serum albumin) to reduce non-specific binding of antibodies followed by unlabeled avidin and biotin to inhibit non-specific attachment of fluorescent streptavidin to cellular, primarily mitochondrial, biotin. Then, samples were incubated in the presence of 5  $\mu$ g/ml OP15 mAb against

an intracellular epitope of ErbB2, AlexaFluor546-trastuzumab and 5  $\mu\text{g/ml}$  biotinylated HABC (hyaluronic acid binding complex) overnight. Trastuzumab was purchased from Roche and labeled with AlexaFluor546 or AlexaFluor647 according to the instructions of the manufacturer. Afterwards, sections were labeled with AlexaFluor488-streptavidin and AlexaFluor647 goat anti-mouse IgG for 60 min.

Image acquisition was carried out on an Olympus FV1000 confocal microscope using a 60 $\times$  oil immersion objective (N.A.=1.35). AlexaFluor488, AlexaFluor546 and AlexaFluor647 were excited by the laser lines at 488 nm, 543 nm and 633 nm, respectively. Fluorescence emissions of the three dyes were collected in the spectral regions of 515 $\pm$ 15 nm, 590 $\pm$ 35 nm and 705 $\pm$ 50 nm ensuring minimal spectral crosstalk.

### ***3.8. Preparation of oligonucleotide-labeled proximity probes***

Primary antibodies at a concentration of 4 mg/ml in PBS were conjugated for two hours at room temperature with a 30-fold molar excess of succinimidyl 4-hydrazinonicotinate acetone hydrazone (SANH). A 3.5-fold molar excess of aldehyde-modified priming and non-priming oligonucleotides were conjugated for three hours with SANH-conjugated antibodies (priming and non-priming oligonucleotides). The concentration of selected pooled fractions was estimated by gel electrophoresis and binding of oligonucleotide-conjugated primary antibodies to cells was checked by immunofluorescence.

### ***3.9. Labeling of cells for in situ PLA***

One million freshly harvested cells were washed in cold PBS and fixed in 1% formaldehyde for 10 minutes on ice. After washing twice in cold PBS they were incubated at 37°C in blocking buffer for 30 minutes. After centrifugation and

removal of the blocking buffer the cells were labeled with proximity probes and fluorescent antibodies at a concentration of 10-20  $\mu\text{g/ml}$  dissolved in antibody diluent for 1-2 hours. In order to remove unbound proximity probes the cells were washed twice in TBST followed by incubation with connector oligonucleotides for 15 min. After washing in TBST the samples were incubated with Duolink Ligation solution for 15 min followed by washing in TBST and incubation with Duolink Amplification solution for 60 min. Afterwards the cells were washed once in TBST and the product of the rolling circle amplification reaction was visualized by adding 25 nM Cy5-labeled detector oligonucleotide dissolved in detection buffer. Finally, the cells were washed in TBST followed by fixation in 1% formaldehyde.

### ***3.10. Measurement and analysis of PLA***

A FACSCalibur flow cytometer was used to analyze cells labeled with proximity probes and fluorophore-conjugated antibodies. The Cy5 signal of the oligonucleotide used to detect the PLA signal was excited at 635 nm and measured in the FL4 channel through a 661/16 nm band-pass filter. The fluorescence of AlexaFluor488-conjugated antibodies was excited at 488 nm and recorded in the FL1 channel through a 530/30 band-pass filter. Data of 20,000 cells recorded in list-mode format was analyzed by the ReFlex software.

## 4. Results and discussion

### 4.1. Investigation of the mechanism of action of elisidepsin

#### 4.1.1. Expression of ErbB1-3 does not alter the sensitivity of CHO and A431 cells to elisidepsin

In order to investigate the role of ErbB2 and ErbB3 in elisidepsin sensitivity we have established two CHO-derived cell lines stably expressing ErbB2 (CHO-ErbB2) or ErbB2 and ErbB3 (CHO-ErbB2-3). To examine the long-term effect of elisidepsin, cell lines were treated with different concentrations of elisidepsin for 2 hours and the cells were allowed to grow for 3 days in the absence of the drug. The three cell lines were found to be equally sensitive to elisidepsin (IC<sub>50</sub> in CHO cells: 10±0.7µM; IC<sub>50</sub> in CHO-ErbB2 cells: 10.5±0.9µM; IC<sub>50</sub> in CHO-ErbB2-3 cells: 10.3±0.8µM). In order to analyze the short-term effect of elisidepsin on viability and to investigate the time course of elisidepsin-induced killing we followed the uptake of PI in the presence of different concentrations of the drug for one hour. Both the extent and kinetics of elisidepsin-induced membrane permeabilization were comparable in the three cell lines.

To confirm the lack of effect of ErbB protein expression on elisidepsin sensitivity in another cell line we checked the short- and long-term effects of the drug on A431 cells and its subclones: A4erbB1, A4erbB2 and A4erbB3 cells. Similar to CHO cells introduction of ErbB proteins (ErbB1-3) into A431 did not change the long-term sensitivity of cells to elisidepsin (IC<sub>50</sub> in A431: 8.2±0.8µM; IC<sub>50</sub> in A4erbB1: 9.4±0.5µM; IC<sub>50</sub> in A4erbB2: 9.9±1.0µM; IC<sub>50</sub> in A4erbB3: 9.9±0.9µM). The short-term effectiveness of elisidepsin was not altered by overexpression of any of the ErbB proteins either. We concluded that the elisidepsin-induced cytotoxic effect is a rapid event and the expressions of ErbB1, ErbB2 and ErbB3 do not influence the sensitivity of cells to the drug.

#### *4.1.2. Elisidepsin decreases the homoassociation of ErbB2 and ErbB3*

The direct or indirect involvement of a protein in the action of a drug is often reflected in the altered association state of the protein. Since the previous results imply that ErbB3 is affected by elisidepsin, we tested whether the homo- and heteroassociations of ErbB2 and ErbB3 are altered by elisidepsin treatment. Flow cytometric FRET measurements revealed that while the homoassociations of both ErbB2 and ErbB3 were significantly decreased by elisidepsin, their heteroassociation did not change. These findings imply that the observed changes in the homoassociations of ErbB2 and ErbB3 are indirectly linked to the mechanism of action of elisidepsin although neither ErbB2 nor ErbB3 is necessary for elisidepsin sensitivity.

#### *4.1.3. Elisidepsin induces the redistribution of ErbB3 and GPI-anchored proteins into the intracellular space*

FRET measurements are sensitive for the interactions of proteins on the molecular scale, but the distribution of molecules and their clustering on the micrometer scale are not revealed. In order to show the effect of elisidepsin on this latter dimension of associations control and elisidepsin-treated CHO-ErbB2-3 cells were fixed, permeabilized and stained with fluorescent antibodies against ErbB2 and ErbB3. Confocal microscopy showed that elisidepsin did not change the distribution of ErbB2, while that of ErbB3 was significantly altered in that the drug induced the redistribution of the protein from the plasma membrane to the intracellular space. To corroborate the selective effect of elisidepsin on the distribution of ErbB3 we investigated A431 cells stably transfected with one of ErbB1-3 fused to spectral variants of GFP. While elisidepsin did not affect the typical membrane localization of ErbB1 and ErbB2, ErbB3 accumulated intracellularly upon treatment. Since lipid rafts were also implicated in the

mechanism of action of elisidepsin, we investigated the effect of the drug on the distribution of GPI-eGFP transiently transfected to A431 cells. GPI-eGFP was present both in the plasma membrane and in intracellular vesicles in untreated cells, but an almost exclusive localization in vesicles was observed after elisidepsin treatment. These results imply that elisidepsin selectively induces the redistribution of GPI-anchored proteins and ErbB3 from the plasma membrane.

#### *4.1.4. Elisidepsin induces abrupt changes in the order of the plasma membrane*

The finding that elisidepsin exerts many effects on the interactions and distribution of ErbB proteins is in contrast with the lack of effect of ErbB protein overexpression on elisidepsin sensitivity. We attempted to relieve this apparent contradiction by assuming that all the changes induced by elisidepsin, including the redistribution of membrane proteins and permeabilization, are the consequences of primary membrane effects caused by the drug. We used two fluorescent probes. The fluorescence anisotropy ( $r$ ) of TMA-DPH specifically reports on the microviscosity of the plasma membrane since it cannot cross the cell membrane and enter the cytosol. Treatment of A431 cells with 10  $\mu$ M elisidepsin induced an almost instantaneous decrease in the fluorescence anisotropy of TMA-DPH indicating an increased membrane fluidity followed by a gradual and incomplete return of anisotropy to the initial value in  $\sim$ 20 min. The generalized polarization (GP) of Laurdan is a sensitive measure of the order of the plasma membrane and of the extent of penetration of water molecules into the plasma membrane. The GP of Laurdan was already increased by elisidepsin in one minute, it peaked at  $\sim$ 2 min and gradually and partially declined toward the initial value in  $\sim$ 20 min. The increased GP of Laurdan indicates a higher order of the plasma membrane and a restricted access of water to Laurdan in the plasma membrane.

In order to establish that the observed membrane effects of elisidepsin are not unique to the A431 cell line we repeated the experiments with SKBR-3 cells displaying higher sensitivity to elisidepsin. The overall tendency of the elisidepsin-induced changes in the  $r$  of TMA-DPH and in the GP of Laurdan in SKBR-3 cells was similar to that observed in A431 cells. The abrupt and specific changes in the order and fluidity of the lipid bilayer support our assumption that the primary target of elisidepsin is the plasma membrane.

## ***4.2. Inhibition of trastuzumab binding with hyaluronan in human tissue samples***

### ***4.2.1. Trastuzumab binding is inhibited in patients with high hyaluronan production***

We developed a method for the quantitative determination of the binding of trastuzumab normalized to ErbB2 expression in human breast cancer tissue samples. Sections were triple-stained for ErbB2 and hyaluronan expressions and with trastuzumab. Hyaluronan expression was measured by HABC staining, while a monoclonal antibody recognizing an intracellular epitope of ErbB2 was used to quantitate ErbB2 levels (mAb OP15). Cell membranes were identified in the images (segmentation) and the intensity of trastuzumab normalized to ErbB2 expression on a pixel-by-pixel basis was calculated in the cell membrane. The pericellular density of hyaluronan was also determined in the membrane mask. The mean of the trastuzumab/OP15 ratio was determined for every patient based on ~4 sections with ~4 images taken from every tissue section. The normalized trastuzumab binding showed a remarkable negative correlation with pericellular hyaluronan density with a correlation coefficient of -0.52. The square of the correlation coefficient is 0.27 implying that approximately a quarter of the variance of normalized trastuzumab binding is accountable by pericellular hyaluronan

density. The samples were divided into three groups (low, medium and high hyaluronan density). The mean normalized trastuzumab binding was more than 2-times higher in samples with low hyaluronan content than in those showing high hyaluronan density. These data present strong evidence for pericellular hyaluronan inhibiting the binding of trastuzumab to ErbB2.

#### *4.2.2. FRET does not artificially introduce the negative correlation between trastuzumab binding and pericellular hyaluronan density*

Several factors could generate the impression of a negative correlation between the binding of two fluorescent probes. FRET-induced quenching of AlexaFluor488-tagged HABC by AlexaFluor546-trastuzumab would lead to low HABC fluorescence intensity in pixels with high trastuzumab binding. Alternatively, quenching of the fluorescence of AlexaFluor546-trastuzumab by OP15 labeled by AlexaFluor647 would also lead to an artifactual decrease in the trastuzumab/OP15 fluorescence intensity ratio. If any of the above factors would have any effect in the generation of the observed negative correlation between normalized trastuzumab binding and pericellular hyaluronan density, then swapping of the fluorescent labels of trastuzumab and OP15 antibodies would change the correlation. Therefore, we selected one sample from each of the low, medium and high hyaluronan groups and compared their trastuzumab/ErbB2 ratios when labeled with AlexaFluor546-trastuzumab and AlexaFluor647-OP15 (“forward” labeling) and when labeled with AlexaFluor546-OP15 and AlexaFluor647-trastuzumab (“reverse” labeling). We did not observe any significant difference in the tendency of the trastuzumab/ErbB2 ratio between the two labeling conditions. In addition, we have analyzed FRET between AlexaFluor546-trastuzumab and AlexaFluor647-labeled OP15 in cultured SKBR-3 breast cancer cells by flow cytometry. The almost complete absence of FRET between these two epitopes is in accordance with the large distance between

the two antibodies due to their binding to opposite domains (intracellular vs. extracellular) of ErbB2 and argues against an influence of FRET on normalized trastuzumab binding. Therefore, we conclude that the observed negative correlation between fluorescence intensities reflects the negative correlation between the binding of the fluorescent probes.

#### *4.2.3 The negative correlation between trastuzumab binding and hyaluronan density is typically present within a single specimen*

The aforementioned observation of low binding of trastuzumab in the presence of a high local density of pericellular hyaluronan was made when comparing samples from different patients. We also analyzed the same correlation within single specimens by inspecting the two-dimensional histograms of normalized trastuzumab binding vs. hyaluronan density. The negative correlation between trastuzumab binding and pericellular hyaluronan concentration was present in  $\sim 2/3$  of the samples. The effect size was significantly lower in this case than in the patientwise data set in accordance with the lower correlation coefficient ( $r = -0.3$ ) and with the less than 1.5-fold difference between the normalized mean trastuzumab binding in pixels with the lowest and highest hyaluronan densities. The data confirm that pericellular hyaluronan inhibits the binding of trastuzumab to ErbB2.

### ***4.3. Quantitative properties of the PLA method***

#### *4.3.1. Initial observations on the lack of correlation between FRET and PLA*

As an initial approach to the comparison of PLA and FRET results we compared the two methods in detecting EGF-induced increased homoassociation of ErbB1. While the homocustering of ErbB1 was significantly increased by EGF according to FRET results on cells labeled by two different antibodies, PLA did not reveal any significant change in the homoassociation of the receptor upon growth factor

stimulation. Next, we analyzed the correlation between FRET and PLA results obtained from intermolecular and intramolecular measurements with another member of the ErbB family, ErbB2. Cells were labeled with donor- and acceptor-conjugated antibodies against different epitopes of ErbB2 to measure intramolecular FRET. Next, cells were labeled with donor-conjugated and acceptor-conjugated anti-ErbB2 antibodies against the same epitope of the protein to analyze homoassociation. The FRET efficiency was significantly higher when measuring intramolecular distances, while a different tendency was observed in the case of PLA measurements. The distributions of immunofluorescence labeling and the PLA signal were in accordance with published data.

#### *4.3.2. Different dependence of FRET and PLA on expression levels when measuring ErbB2 homoassociations*

Given the lack of correlation between FRET and PLA results we compared the dependence of FRET and PLA on the density of the fluorophore-tagged or oligonucleotide-conjugated antibody. SKBR-3 cells were labeled with a mixture of trastuzumab-priming and trastuzumab-non-priming antibodies to analyze the homoassociation of ErbB2. Cells were also labeled with a fluorescent antibody against ErbB2 to measure the expression level of the protein. An obvious saturation phenomenon was observed at high, albeit not extreme, expression levels of ErbB2. The PLA signal may be saturated at expression levels often observed in cancer cells. The tendency of the PLA signal to saturate at high expression levels was observed at three different ratios (1:3, 1:1, 3:1) of the antibodies labeled with priming and non-priming oligonucleotides.

In striking contrast, the FRET intensity for ErbB2 homoassociation was not saturated. Since the FRET intensity is proportional to both the amount of the fluorophore-labeled antibodies ( $I_d$ ) and their proximity ( $E$ ) it is analogous to the

PLA signal. The fact that the dependence of the FRET intensity and the PLA signal on the expression level of the investigated protein is different suggests that the two phenomena reveal protein associations in distinctly different ways.

#### *4.3.3. FRET and PLA exhibit different dependence on expression levels in the case of intramolecular measurements*

The proximity between two epitopes of the same molecule is expected to provide a consistent point of reference for comparing the reliability of PLA and FRET measurements since intramolecular distances do not depend on expression levels. Therefore, the proximity between the epitopes of two anti-ErbB2 antibodies, pertuzumab and trastuzumab, was analyzed by PLA and FRET. Similar to the aforementioned findings intramolecular PLA exhibited saturation which was apparent in the brightest quarter of the fluorescence intensity distribution. In contrast, FRET intensity showed a strong linear correlation with the expression level and the FRET efficiency was constant at all expression levels when measuring the proximity of the same epitopes using FRET. We concluded that the saturation phenomenon in PLA manifests itself in intra- and intermolecular measurements as well.

#### *4.3.4. Antibody density distinctly influences FRET and PLA measurements*

In order to adjust the antibody density on the cell surface in a controlled way and measure the dependence of PLA on antibody density cells were labeled with pertuzumab-non-priming, trastuzumab-priming and A488-trastuzumab antibodies. The total concentration of trastuzumab-priming and A488-trastuzumab antibodies was kept constant, but the ratio of trastuzumab-priming was changed from 100% to 10%. The PLA signal saturated when plotted as a function of the density of the trastuzumab-priming antibody. A similar experiment was performed using FRET as well. The pertuzumab-binding epitope of ErbB2 was saturated with donor-

conjugated antibodies, while acceptor-tagged and unlabeled antibodies competed for the trastuzumab binding epitope. The FRET efficiency showed a strict linear dependence on the density of the acceptor-conjugated antibody. We concluded that the saturation phenomenon is reproducibly observed in PLA experiments.

*4.3.5. Saturation effects in PLA are not significantly influenced by the duration of rolling circle amplification*

We tested whether the saturation phenomenon in PLA is related to depletion of nucleoside-triphosphate substrates during rolling circle amplification. Therefore, we analyzed the dependence of the PLA signal on the expression level at three different incubation times for rolling circle amplification (30, 90 and 150 min). The PLA signal increased with the incubation time, albeit not linearly. Although the degree of saturation tended to increase with the incubation time, it was obviously present in the case of the shortest incubation time as well. We concluded that substrate depletion during the amplification process in PLA cannot account for the saturation effects described above.

## 7. Summary

### 7.1. Investigations in the mechanism of action of elisidepsin

Elisidepsin is a marine-derived anti-tumor agent with unique mechanism of action. We investigated the effect of the drug on the distribution of ErbB proteins and systematically compared the elisidepsin sensitivity of cell lines overexpressing ErbB receptors. The fact that the elisidepsin sensitivity of CHO- and A431-derived cell lines did not correlate with their ErbB protein expression levels argues against a decisive role of ErbB receptors in determining elisidepsin responsiveness. In particular, the finding that overexpression of ErbB2 or ErbB2 and ErbB3 in CHO cells did not alter elisidepsin sensitivity is a strong evidence for the lack of involvement of ErbB2 and ErbB3 in conferring elisidepsin sensitivity. Flow cytometric FRET investigations revealed that elisidepsin induces a decrease in the homoassociations of ErbB2 and ErbB3 without measurable changes in their heteroassociations. On the other hand, GPI-anchored proteins and ErbB3 were preferentially redistributed upon elisidepsin treatment from the plasma membrane to intracellular vesicles. We did not observe the internalization of every kind of membrane protein since ErbB1 and ErbB2 persisted in the plasma membrane after elisidepsin treatment. We demonstrated that although elisidepsin induced changes in the homoassociation and cellular distribution of ErbB and GPI-anchored proteins, its cytotoxic effect was independent of the expression of ErbB1-3. The changes in the fluorescence anisotropy of TMA-DPH and in the GP of Laurdan immediately after application of the drug preceded cell death and membrane permeabilization. The primary target of elisidepsin is the plasma membrane and all other effects are the consequences of the primary membrane effects.

## ***7.2. Trastuzumab binding is inhibited in patients with high hyaluronan production***

ErbB2 is a therapeutical target in the treatment of breast cancer. Although in combination with chemotherapy trastuzumab is an efficient and well-tolerated drug, primary and acquired resistance is a challenging problem. Since overproduction of hyaluronan has already been shown to lead to poor prognosis in breast cancer, we investigated ErbB2-overexpressing breast cancer tissue samples and correlated trastuzumab binding with the local density of hyaluronan. The demonstration of the anticorrelation between hyaluronan density and normalized trastuzumab binding was made possible by quantitative image analysis and by limiting the evaluation of pixel intensities to the cell membrane. We found that the normalized binding of trastuzumab showed a negative correlation with the pericellular density of hyaluronan. The current findings are in agreement with our previous *in vitro* and mouse xenograft experiments results. The mechanism of how hyaluronan inhibits trastuzumab binding is unknown. It is possible that the dense hyaluronan meshwork directly covers or masks the trastuzumab binding epitope of ErbB2. Alternatively, hyaluronan has been implicated as one of the factors leading to increased interstitial pressure in malignant tumors which results in blood vessel collapse and inhibition of pressure difference-driven transport of drug molecules to the center of the tumor. The demonstration that hyaluronan depletion results in improved tumor penetration of conventional chemotherapeutic drugs, liposomes and trastuzumab in animal models argues in favor of hyaluronan being an important factor limiting the accessibility of drug binding sites in cancer. Although hyaluronan is by no means the only factor contributing to trastuzumab resistance, our results have important clinical implications and call for testing the hyaluronan level of ErbB2-overexpressing tissue samples and warrant further development of drug candidates lowering tissue hyaluronan levels.

### 7.3. Quantitative properties of the PLA method

Although the recent introduction of proximity ligation assay (PLA) into the armamentarium of proteomics opened new possibilities in the investigation of protein interactions, PLA has not been evaluated in a quantitative way. Some discrepancies between PLA and other well established techniques prompted us to compare the results of PLA and fluorescence resonance energy transfer (FRET). The investigated proteins were labeled with oligonucleotide- or fluorophore-conjugated antibodies and their interactions were analyzed by flow cytometry in order to obtain statistically robust data. The PLA signal for the homoassociation of a membrane protein, ErbB2, reached saturation when plotted against the expression level. Both intermolecular and intramolecular PLA signals reached saturation at high expression levels. In contrast, the FRET efficiency was independent of, while the FRET signal exhibited a strict linear correlation with the expression levels of proteins. When the density of oligonucleotide- and fluorophore-conjugated antibodies was systematically changed by competition with unlabeled antibodies the FRET signal was linearly proportional to the amount of bound fluorophore-tagged antibodies, whereas the PLA signal was again saturated. The saturation phenomenon in PLA could not be eliminated by decreasing the duration of the rolling circle amplification reaction. Our data imply that PLA is a semiquantitative measure of protein colocalizations due to non-linear effects in the reaction and that caution should be exercised when interpreting PLA data in a quantitative way.

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## 6. Publications



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Candidate: Tímea Váradi

Neptun ID: E3LY9G

Doctoral School: Doctoral School of Molecular Medicine

### List of publications related to the dissertation

1. **Váradi, T.**, Mersich, T., Auvinen, P., Tammi, R., Tammi, M., Salamon, F., Besznyák jr., I., Jakab, F., Baranyai, Z., Szöllősi, J., Nagy, P.: Binding of trastuzumab to ErbB2 is inhibited by a high pericellular density of hyaluronan.  
*J. Histochem. Cytochem.* "accepted by publisher", 2012.  
IF:2.381 (2010)
2. Mocanu, M., **Váradi, T.**, Szöllősi, J., Nagy, P.: Comparative analysis of fluorescence resonance energy transfer (FRET) and proximity ligation assay (PLA).  
*Proteomics.* 11 (10), 2063-2070, 2011.  
DOI: <http://dx.doi.org/10.1002/pmic.201100028>  
IF:4.815 (2010)
3. **Váradi, T.**, Roszik, J., Lisboa, D., Vereb jr., G., Molina-Guijarro, J.M., Galmarini, C.M., Szöllősi, J., Nagy, P.: ErbB protein modifications are secondary to severe cell membrane alterations induced by elisidepsin treatment.  
*Eur. J. Pharmacol.* 667 (1-3), 91-99, 2011.  
DOI: <http://dx.doi.org/10.1016/j.ejphar.2011.05.064>  
IF:2.737 (2010)





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#### List of other publications

4. Szikra, D., Váradi, T., Nagy, I.P.: Preliminary investigations for the application of composite electrodes in organic electrosynthesis.  
*J. New Mat. Electr. Sys.* 11 (4), 273-279, 2008.  
IF:0.67

**Total IF: 10.603**

**Total IF (publications related to the dissertation): 9.933**

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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